The Effect of Octopamine, Cyclic Adenosine 3', 5'-Monophosphate and Calcium on Protein Phosphorylation in *Schistocerca gregaria* Central Nervous System

by

Dino Rotondo

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Department of Biochemistry,
University of Glasgow,
GLASGOW.
G12 8QQ

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Abbreviations

The abbreviations used in this thesis are those recommended in the Biochemical Journal publication "Policy of the Journal and Instructions to Authors " revised 1984, (Biochem. J. 217, 1-27), except for the following;

AP	action potential
CNS	central nervous system
DA	dopamine
epsp	excitatory postsynaptic potential
ipsp	inhibitory postsynaptic potential
M_r	relative molecular weight
NA	noradrenaline
Pf	particulate fraction
SDS	sodium dodecyl sulphate
Sf	soluble fraction
TCA	trichloroacetic acid, % quoted is always w/v
5-HT	5-hydroxytryptamine (serotonin)

Table of Contents

Table of Contents (Continued)

Table of Contents (Continued)

Table of Contents (Continued)

Table of Contents (Continued)

Table of Contents (Continued)

List of Figures

List of Figures (Continued)

List of Figures (Continued)

List of Tables

SUMMARY

The effects of octopamine (a putative neurotransmitter) cyclic AMP and calcium on the phosphorylation of protein from the CNS of the desert locust, (Schistocerca gregaria) were investigated.

Two main approaches were used to study protein phosphorylation

a) Intact cerebral ganglia were incubated with ^{32}Pi and

b) Homogenates of cerebral ganglia were incubated with [$\gamma^{32}P$] ATP.

The incorporation of ^{32}P into total protein and specific protein was measured in both cases. The ^{32}P bound to total protein was measured by precipitating the proteins with TCA and measuring the radioactivity remaining after treatment of the acid insoluble residue with acidified chloroform/methanol and ribonuclease. The ^{32}P bound to specific proteins was measured by solubilizing the proteins with SDS and separating them by electrophoresis on 10% polyacrylamide/SDS gels followed by autoradiography.

In intact cerebral ganglia octopamine (10^{-4} M) stimulated an increase in the phosphorylation of total protein within 10 minutes. This subsequently decreased to the control level between 3 and 6 hours. A second octopamine-stimulated increase was observed after 22 hours. The measurement of specific protein phosphorylation after 10 minutes and 22 hours incubation with octopamine revealed that it stimulated the phosphorylation of a protein (M_r 39,000). The phosphorylation of 2 other proteins (M_r 21,000 and 25,000) occured after 22 hours which did not occur after 10 minutes.

Octopamine also stimulated the phosphorylation of the M_r 39,000 protein in homogenates of cerebral ganglia but only under conditions which favoured the elevation of cyclic AMP levels prior to the measurement of protein phosphorylation.

Dibutyryl cyclic AMP (10^{-3} M) stimulated the phosphorylation of the M_r 39,000 as well as at least 8 other proteins in intact tissue and the pattern of phosphorylation was similar to that of cyclic AMP in homogenized tissue.

Cyclic AMP (10^{-5} M) stimulated the phosphorylation of many proteins in homogenates including the M_r 39,000 protein within 10 seconds. The major proportion of cyclic AMP-dependent protein phosphorylation resided in the soluble fraction, although phosphorylation of the M_r 39,000 protein occured in both soluble and particulate fractions. Cyclic AMP-dependent protein kinase activity was maximal at a cyclic AMP concentration of 50 µM and half-maximal at 2.5 µM with a Km of 50 µM ATP and its time course reached a maximum within 60 seconds with 10 µM cyclic AMP and 10 µM ATP.

Calcium also stimulated total protein phosphorylation in homogenized tissue. A maximum was achieved in the presence of 700 µM EGTA with a $CaCl_2$ concentration of 1 mM and half-maximal at 400 µM. Calcium (1 mM) stimulated the phosphorylation of 3 proteins (M_r 62,000; 54,000 and 31,000) in crude homogenate. In the soluble fraction calcium (1 mM) only stimulated the phosphorylation of the M_r 54,000 protein no stimulation by

calcium alone was observed in the particulate fraction. Incubation of the particulate fraction with calcium (1 mM) and calmodulin (10 µg/ml), however, resulted in the phosphorylation of 3 proteins, (M_r 62,000; 54,000 and 31,000).

Cyclic AMP-dependent protein kinase inhibitor prevented the cyclic AMP-dependent phosphorylation of all proteins from crude homogenate, soluble and particulate fractions. In addition, it also prevented the calcium-dependent phosphorylation of proteins from the crude homogenate and soluble fraction and calcium/calmodulin-dependent protein phosphorylation from the particulate fraction.

1 INTRODUCTION

1:1 An assessment of the Invertebrate Nervous System

The central nervous system of insects and other invertebrates has been a favoured subject for research by 2 main groups; Pesticide Scientists and Neurobiologists.

Pesticide Scientists study the CNS of both vertebrates and invertebrates to see if there are any differences which would allow pesticides to be highly selective. Neurobiologists study the invertebrate CNS, either in its own right, or in comparison to the mammalian CNS, in the hope that this will allow the simpler system to be used as a model for the more complex mammalian system. Invertebrate nervous systems are simpler in that they contain relatively fewer nerve cells than mammalian CNS. This may make them useful as models, i.e. the experimental use of a lower, less complex animal, from which valid conclusions can be drawn about the structure and function of higher animals, (Strang, 1981).

One of the most important parts of our knowledge of the basic mechanism of neuronal function, the action potential, has been obtained in an invertebrate preparation, the giant axon of the squid. With this preparation, the ionic basis of electrical activity in neurones was elucidated, (Hodgkin et al, 1949; Hodgkin, 1951). To date, there has been no exception to the observation, that the fundamental mechanism of neuronal activity i.e. depolarisation, impulse propagation and neurotransmitter release in response to, Na^+, K^+, Cl^- and Ca^{2+} ion fluxes is the same in both vertebrates and invertebrates.

Recently, the invertebrates have been used to investigate the molecular and cellular basis of behaviour, as they are considered by some to have behavioural complexity, yet anatomical simplicity, (Hoyle, 1975). Studies of the gastropod mollusc, Aplysia californicus, show that small systems of neurones are capable of forms of learning and memory, (Kandel, 1979).

The gill withdrawal reflex of Aplysia is a behavioural response to stimulation of the siphon. This is controlled by 24 sensory neurones, which make contact with 6 gill motorneurones and at least one inhibitory cell. This reflex exhibits 2 forms of learning; habituation and sensitization. Habituation is a decreased behavioural response to an initial stimulus which is presented repeatedly. It is thought to be the first learning process in human infants and it gives rise to both short- and long-term memory. Sensitization is a form of learning and memory where the response to a particular stimulus is enhanced when it is followed by a more noxious stimulus. As with habituation, sensitization can be either short- or long-term and is an independent and opposite form of learning. From these experiments on Aplysia, elementary aspects of what are regarded as mental processes have been found to reside in the activity of a small number of interlinked neurones, (Kandel, 1979).

Just as the electrophysiological properties of all neurones seem to be almost identical, the underlying biochemical properties also appear to be similar. Biochemically the action potential is thought to be supported by the electrogenic pump

the Na^+- and K^+-dependent ATPase. Its characteristics seem well conserved from insects to mammals. The affinity for ATP, and requirements for Na^+, K^+ and Mg^{2+} of solubilized Na^+-K^+-dependent ATPase from frozen housefly heads are similar to those of mammalian Na^+, K^+-dependent ATPases, (Jenner & Donnellan, 1976). The second messengers which mediate the actions of compounds on the cell surface and translate them into intracellular responses are also identical in vertebrate and invertebrate. Cyclic AMP and GMP, and ions such as Ca^{2+}, K^+ and Na^+, are not only present in all nervous systems but are ubiquitous in all organisms from bacteria to mammals and appear to have the same functions. It has been proposed that cyclic AMP metabolism in mammals has evolved from lower organisms, and that neurotransmitters may have been intracellular mediators in primitive cells. It is suggested that they were possibly involved in transducing information in relation to amino acid metabolism, as all the putative neurotransmitters are either derived from amino acids (acetylcholine and the catecholamines), or are amino acids themselves (glycine). It has also been suggested that the evolution of the nervous system was preceded by that of the neurotransmitters, as some transmitter substances such as acetylcholine occur in lower organisms, with no apparent neuronal function, (Tomkins, 1975).

1:2 Advantages of the Invertebrate Nervous System

One of the main reasons that invertebrate CNS has been favoured by electrophysiologists is that individual neurones are large in comparison to those in the vertebrate CNS, typically larger than 900 μm, in some molluscs, (Giacobini, 1969), compared to the 8-25 μm range in rat cerebral cortex, (Rose, 1967). The larger neurones facilitate both intracellular potential recording and the microapplication of drugs. There are also far fewer neurones than in a vertebrate tissue of the same size, human brain is reported to have upward of 10^{10} nerve cells in a 0.2 mm thick segment of the granular layer of the cerebral cortex, (Bullock, 1975). The brains of the honey bee (Apis mellifera) and housefly (Musca domestica), on the other hand, contain totals of 8.5×10^5 and 3.3×10^5 neurones respectively (Willhoft, 1967 ; Strausfeld, 1975). The neurones of human brain also contain 10^9 more cell to cell junctions than the above insects and with present day techniques, this would make the human brain almost impossible to investigate in terms of neurone to neurone signalling.

Because of the much lower number of cells and their larger size many invertebrates possess readily identifiable neurones. A detailed map has been produced of more than 60 identified neurones in the metathoracic ganglion of the desert locust (Schistocerca gregaria), using electrophysiological and iontophoretic staining techniques, (Hoyle, 1975). These advantages which attract the electrophysiologist, however, do not always facilitate the job of the biochemist. The most obvious disadvantage being that invertebrate preparations, especially

insects generally yield only small amounts of tissue. There is also the added drawback,that when insect ganglia are dissected, this ruptures the tracheal system which permeates all the nervous tissue in situ. The tracheal system normally supplies oxygen directly to the tissue and rupture would result in a diminished supply of oxygen to the tissue. This can be offset, to an extent, by increasing the dissolved oxygen concentration in the bathing medium, (Clement & Strang, 1978).

These apart there are additional advantages for the biochemist in choosing the invertebrate CNS. Unlike mammalian CNS, invertebrate nervous tissue possesses considerable mechanical strength and functions well in vitro. Insect ganglia can generate spontaneous electrophysiological activity for a considerable length of time, (Clement & Strang, 1978). There is no vascular system present in invertebrate nervous tissue, although they do possess a "blood-brain" barrier analogous to that in mammals.

Because of the large size of some invertebrate neurones, it is possible to carry out microchemical analysis on individual neurones, and correlate this to electrophysiological activity. The stretch receptor neurone of crustacea has been used extensively as a model for biochemical and pharmacological studies in single cells. This neurone is one of the rare examples of a neurone which is completely isolated from the rest of the nervous system. It displays all the electrical characteristics and properties of nerve cells in general. In the stretch receptor

neurone of the crayfish, it has been possible to apply chemical, electrical and physiological (stretch) stimuli and measure metabolism. Enzyme activity and oxygen uptake have been measured between periods of impulse activity recording, (Giacobini et al, 1963) and also changes in pyridine nucleotide levels after prolonged physiological stimulation and at rest using micro-fluorimetric techniques, (Giacobini & Grasso, 1966). This has enabled dynamic changes in oxidation-reduction reactions to be followed under varying experimental conditions.

1:3 Organisation of the Insect Nervous System

1:3:1 General Outline

The desert locust (Schistocerca gregaria), the insect studied in the project will be described and is representative of the class insecta.

The brain in arthropods is defined as the main ganglionic mass superior to the oesophageal canal and consists of the fused ganglia of 3 pre-oral segments. It is the principal association centre of the nervous system as it directly receives information from the sense organs in the head and from ascending interneurones and so initiates and controls persistent behavioural patterns. The 'brain' and a ventral cord of segmentally arranged ganglia, united by paired longtitudinal connectives constitute the central nervous system (Figures 1 and 2). The ganglia can act as processing centres for their individual segment independently of the others in the cord. Overall, the plan is central interneurones and motorneurones located within the ganglia and peripheral sensory (afferent) neurones.

1:3:2 The Ganglion

The ganglia are protected by the neural lamella, which is a non-cellular sheath of colagen-like fibres embedded in a muco-polysaccharide mucoprotein matrix. Internal to the neural lamella, lining the surface of the ganglia is a layer of glial cells forming the perineurium. These cells are rich in fat globules and glycogen granules and carry out the role of storing and transferring nutrients and metabolites to the neuronal cells.

Figure: 1 The Nervous System of S. gregaria in situ

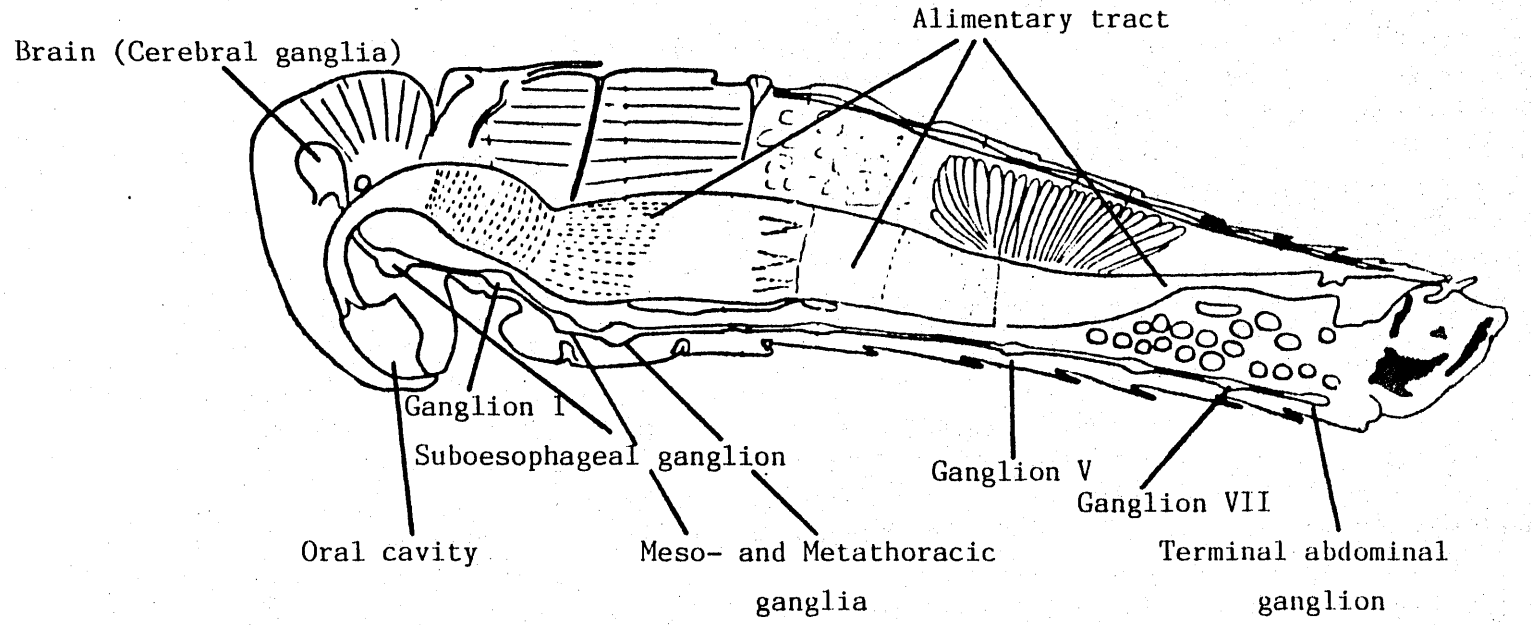
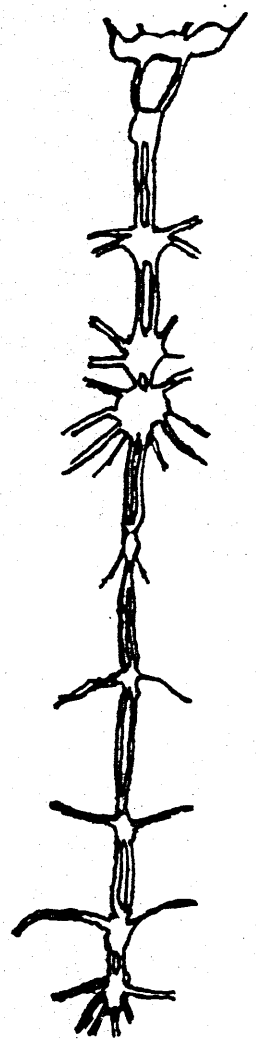


Figure: 2 The Isolated Central Nervous System of S. gregaria



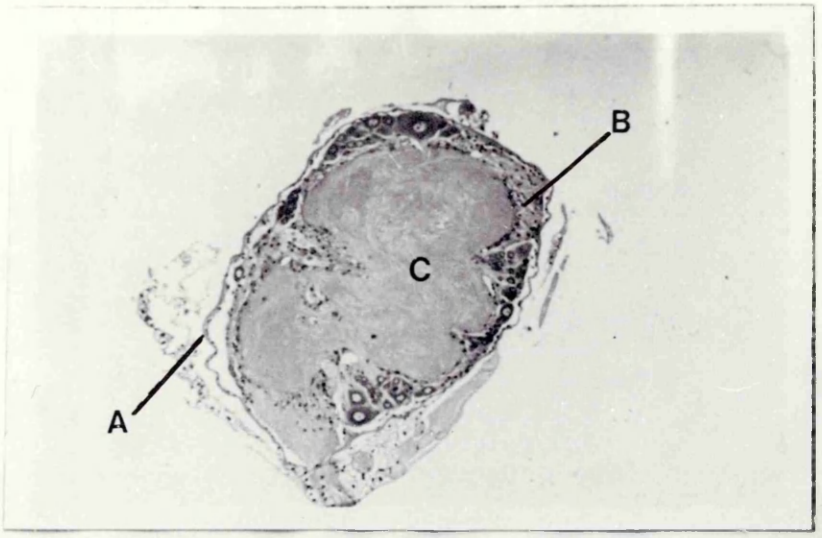
In addition, the perineurium provides a 'blood-brain-barrier' analogous to that of the mammalian CNS. The perineurium ensheaths an array of monopolar pear-shaped neurones. The neuronal cell bodies (perikarya) are located at the periphery, sending their axons to the core of the ganglion where they branch to form a complicated structure called the neuropile, (Figure 3). Each neurone is almost completely ensheathed by one or more glial cells which form a complex structure around the perikarya, but at synapses the glial sheath is characteristically absent. Synapses always occur between nerve processes. No axosomatic synapses have been described in insects, unlike vertebrate nevous systems. Some species, although, have somatosomatic contacts characteristic of electrotonic synapses. There are 2 types of axo-axonic contacts which have been observed;

a) En passent or longtitudinal, where the axon makes contact in passing, sometimes being a single contact or in some cases, the fibres anastomise several times before continuing on their course.

b) Terminal, where axon end knobs make contact with one or more axonic branches.

At the electron microscope level, insect central synaptic endings contain many; small dense-cored vesicles (30-60 nm), larger dense-cored vesicles (60-150 nm), mitochondria,and glycogen particles, as in many vertebrate nervous tissues, (Evans, 1980). It is generally accepted that the small and large dense-cored vesicles of vertebrate nervous tissue contain

Figure: 3 Cross Sections of Cerebral Ganglion

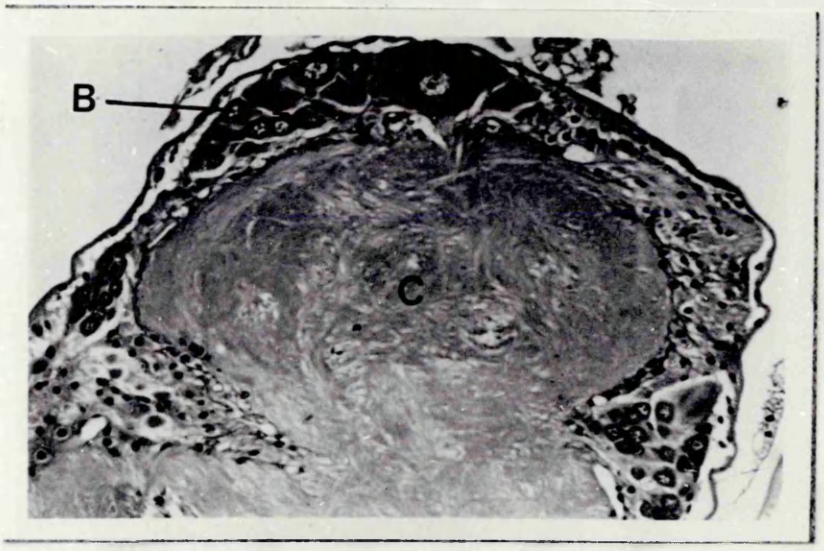


x 48 magnification

A - Neural lamella

B - Perineurial cell layer

C - Neuropile



x145 magnification

Sections prepared by Dr. A.T. Kilpatrick

noradrenaline, (Nelson & Molinoff, 1976). Dense-cored vesicles disappear from mammalian neurones when animals are treated with the amine-depleting drug reserpine, (Hökfelt, 1966). Reserpine does not affect the appearance, size, or distribution of dense-cored vesicles from cockroach brain, (Mancini & Frontali, 1970), which suggests that they do not contain noradrenaline. Electron microscopy of the pre-terminal branches of a neurone which innervates the skeletal muscle of Schistocerca gregaria reveals the presence of numerous large dense-cored vesicles, similar to those of the insect CNS, (Hoyle et al, 1980). It is thought that this neurone is octopaminergic, and octopamine instead of noradrenaline may be stored in the dense-cored vesicles, as octopamine is much more predominant in insect nervous tissue than noradrenaline, (Robertson & Juorio, 1976 ; Evans, 1980).

1:4 Octopamine

An assessment of the distribution of octopamine and its function in both vertebrate and invertebrate species in general and more specifically, its function in locust nervous tissue will be considered.

1:4:1 Structure and History

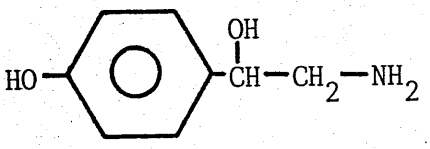
Octopamine is a monophenolic amine whose chemical structure is similar to that of the catecholamines, dopamine, noradrenaline and adrenaline. It differs from noradrenaline in the absence of a hydroxyl group on the benzene ring, (Figure 4). It was first detected in alcohol and acetone extracts from the posterior salivary glands of Octopus vulgaris, (Erspamer, 1948). It was found to have an adrenaline-like effect on the blood pressure and isolated small intestine of rabbits, on the uterus and small intestine of rats and on the frog heart.

1:4:2 Octopamine in Mammals

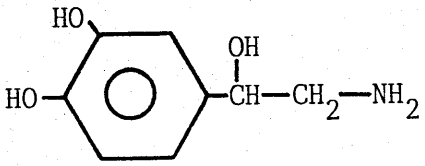
Octopamine is present in the organs and urine from rats, rabbits, and humans. After the heart and kidney, the brain has the highest concentration, (Kakimoto & Armstrong, 1962).

An extremely sensitive enzymatic assay capable of detecting as little as 50 pg of octopamine, was used to study the distribution in various regions of rat central nervous system. The region containing the highest concentration was the hypothalamus, with 13.52 ± 1.78 ng/g of tissue, (Buck et al, 1977). Octopamine has also been shown to be a normal constituent of rat sympathetic

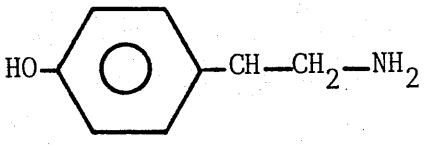
Figure: 4 Structure of Octopamine and related amines



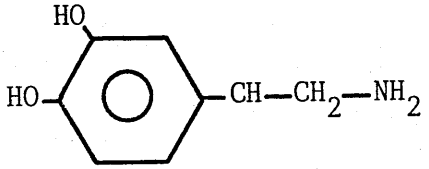
Octopamine



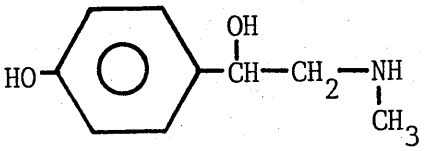
Noradrenaline



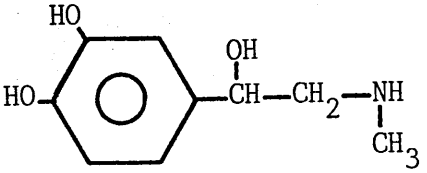
Tyramine



Dopamine



Synephrine



Adrenaline

nerves, (Molinoff & Axelrod, 1969). It is formed *in vivo* from exogenous [^{3}H] tyramine and is released by sympathetic nerve stimulation in the isolated perfused cat spleen, (Snyder *et al*, 1964; Kopin *et al*, 1964). The differential distribution of endogenous octopamine in rat brain and its presence in sympathetic nerves suggests that it might play a role in mammalian synaptic function, although no octopaminergic neurones in mammalian nervous system have been identified. In most organs the content of octopamine is 5-10% that of noradrenaline and it disappears together with noradrenaline after sympathetic denervation. It can also be released from the cat splenic nerve with noradrenaline, thus it may act as a co-transmitter, (Molinoff & Axelrod, 1972). However, in rat salivary gland octopamine does not appear to be present in noradrenergic nerves, (Coyle *et al*, 1974), and the destruction or depletion of central noradrenergic neurones has no effect on the level of octopamine, (Harmar & Horn, 1976).

Octopamine may in some cases be a false transmitter. Aromatic l-amino acid decarboxylase is present in nervous tissue from rat and dog brain stem and can decarboxylate tyrosine to tyramine (the precursor of octopamine), although the favoured pathway in mammalian nervous tissue is hydroxylation, (Lovenberg *et al*, 1962). This would yield small amounts of tyramine which would be converted to octopamine by dopamine β hydroxylase in the noradrenergic storage vesicles and released on stimulation, (Kopin *et al*, 1964). This would account for its relatively low concentration compared with noradrenaline. It has been

proposed that the hypotensive effects of monoamine oxidase (MAO) inhibitors are due to octopamine (which has a much lower pressor effect on blood vessels) being released as a false transmitter. This occurs because tyramine which is normally destroyed by MAO is allowed to be taken up into noradrenergic vesicles and converted to octopamine. Tyramine is found in rat brain, and its concentration in hypothalamus is 11.3 $\pm$ 3.7 ng/g*, (Philips et al, 1974), similar to the concentration of octopamine in this region, (Buck et al, 1977 ; Molinoff & Axelrod, 1972), and intraventricularly injected tyramine is rapidly converted to octopamine, (Wu & Boulton, 1974).

It has been reported that there is an abnormally high urinary excretion of unconjugated p tyramine in patients suffering from schizophrenia, (Boulton, 1971) and Parkinson's disease, (Boulton & Marjerrison, 1972).

In conclusion, it is difficult to assign a role for octopamine in mammals, whether it is physiological or pathological. The fact remains that it is normally present in vertebrate nervous tissue, in relatively small concentrations compared to noradrenaline, which has a well established neurotransmitter role in mammalian nervous system.

*ng/g wet weight of tissue.

1:4:3 Octopamine in Invertebrates

1:4:3:1 Comparison of the Octopamine content of invertebrate and mammalian Nervous Tissue

Although octopamine was first discovered in Octopus vulgaris, it has only recently been associated with a possible physiological role in invertebrates, especially in neural tissue. It has been found in much higher levels in a wide variety of invertebrates than in mammals. Octopamine concentrations in various invertebrates can be measured in the µg/g of tissue range, as opposed to ng/g in mammals. The earthworm (Lumbricus terrestris) contains 8.11 µg/g* in the subpharyngeal ganglion, (Robertson & Juorio, 1976), approximately 1,000 fold greater than in rat brain, (Buck et al, 1977 ; Molinoff & Axelrod, 1972). The highest recorded concentration is in Octopus vulgaris posterior salivary gland, 1.31 mg/g* tissue, (Juorio & Molinoff, 1974). Not only is octopamine in much higher concentrations in invertebrates, but the noradrenaline concentration is generally lower than in mammals. Schistocerca gregaria brain contains a noradrenaline concentration of 110 ng/g* tissue, compared to 490 ng/g* in rat brain. An exception to this trend is the cephalopod class of invertebrates, which also have a high concentration of NA, although they are atypical in other respects. They have high levels of dopamine and 5-HT, but have a lower DA/NA ratio of 3:1 in nervous tissue, (Robertson & Juorio, 1976) compared to the usual invertebrate ratio of 10:1, (Kerkut, 1973).

In all the invertebrate classes investigated with the exception of the cephalopods, the ratio of octopamine to noradrenaline

* /g wet weight of tissue

in the nervous system is greater than 1 i.e. octopamine predominates, whereas the reverse is true in mammals.

1:4:3:2 Phylogenetic Distribution

There seems to be a phylogenetic relationship in the octopamine to noradrenaline ratio, between the protostomes and deuterostomes. Protostomes comprise the phyla of increasingly higher invertebrates flatworms, molluscs, annelids and arthropods, (which consist of the classes crustacea and insecta) where octopamine increasingly predominates over noradrenaline. The reverse is true of the deuterostomes which include the echinoderms, and vertebrates, where the octopamine to noradrenaline ratio is increasingly lower, i.e. noradrenaline becomes more predominant, (Table 1). It has also been observed that this trend occurs in rat brain from the developing foetus through to adulthood, (Table 1). This tends to suggest that there may be a role for octopamine in the developing embryonic nervous system. It has also been speculated that these 2 trends may be further evidence for the 'biogenetic law' put forward by Haeckel, (Robertson & Juorio, 1976), which proposes that the developing embryo passes through the evolutionary steps of that particular species, (Haeckel, 1908).

1:4:3:3 Circulating Octopamine

Haemolymph taken from locusts at rest contains octopamine at a concentration of 30 nM, which increases to 170 nM during the first 10 minutes of prolonged flight, and returns to the resting level, (Goosey & Candy, 1980). This is analogous to the rapid

Table: 1 The Octopamine/Noradrenaline Ratio in Nervous Tissues

Species	Class	Phylum	Tissue	Ratio *OA/NA
Periplaneta americana (cockroach)	Insecta	Arthropoda	Brain	10.4
Schistocerca gregaria (desert locust)	Insecta	Arthropoda	Brain	22.1
Homarus americanus (lobster)	Crustacea	Arthropoda	Brain	>10
Lumbricus terrestris (earthworm)	Oligochaeta	Annelida	Ventral nerve cord	3.8
Helix aspersa (garden snail)	Gastropoda	Mollusca	Brain	1.8
Octopus vulgaris (Mediterranean octopus)	Cephalopoda	Mollusca	Brain	0.3
Loligo vulgaris (squid)	Cephalopoda	Mollusca	Optic lobes	0.2
Pycnopodia helianthoides (starfish)	Asteroidea	Echinodermata	Arm nerve	0.1
Rattus norvegicus (rat)	Mammalia	Vertebrata	Brain, day 15 gestation	1.56
			Brain, day 16 gestation	1.85
			Brain, day 17 gestation	0.39
			Brain, day 22 gestation (birth)	0.04
			Brain, adult	0.01

* OA, octopamine; NA, noradrenaline.

Adapted from Robertson and Juorio, (1976).

rise in plasma catecholamine levels during exercise in man, (Galbo et al, 1975). Octopamine stimulates the oxidation of glucose, trehalose, butyrate and diacyl glycerol in working perfused locust thoracic muscles, as well as strengthening the muscular contractions in concentrations as low as 3×10^{-7}M, (Candy, 1978). It also induces short-term hypertrehalosaemia when injected into the cockroach and is thought to be the mediator of the excitation induced hypertrehalosaemia (EXIT) response, (Downer, 1979).

The origin of this haemolymph octopamine is not known, but possibilities include its release from:

a) Central neurons into the circulating haemolymph to act as a neurohormone.

b) From nerve terminals innervating the flight muscle to act as a local neurohormone, or:

c) From the principle neurohaemal organ in insects the corpus cardiacum.

Extracts of locust corpus cardiacum have little effect on carbohydrate oxidation, (Candy, 1978), and it has a much lower concentration of octopamine than nervous tissues, 0.47 p moles compared to 11.58 p moles in the cerebral ganglion of the locust, (Evans, 1978a). Experimental handling-induced excitation increases haemolymph levels of octopamine and lipid in locusts. Octopamine, when injected into locusts, also increases lipid levels, and both the excitation and octopamine stimulated

hyperlipaemia are attenuated by the adrenergic blocking agent, phenoxybenzamine, (Orchard et al, 1981). This suggests that octopamine has a neurohormonal role in insects under stress.

1:4:3:4 Octopamine in Nervous Tissue

Considerable interest in octopamine has been its possible function as a neurotransmitter, as its highest levels have all been reported in invertebrate nervous tissues, the only exception being octopus salivary gland, (Robertson & Juorio, 1976).

The earliest suggestion of a possible neurotransmitter role for octopamine was made by Carlson, (1968) who showed that synephrine and octopamine were many times more potent than any other adrenergic compound in stimulating luminescence from firefly lantern. Much evidence has been presented to support this view and will be discussed in terms of the criteria which must be fulfilled for a substance to be established as a transmitter, (Phillis, 1970; Storm-Mathieson, 1977), and are listed in Table 2.

1:4:3:4a Localization

Octopamine is present in the nervous tissues of all invertebrate species investigated especially insect, (Evans, 1980). The locust (Schistocerca gregaria) has an octopamine concentration of 2.43 µg/g tissue, and the cockroach (Periplaneta americana) 3.45 µg/g in their cerebral ganglia. Octopamine is also associated with other CNS structures such as the ganglionic chain. Neutral-red a dye which has been used to selectively stain and locate amine containing cells, (Stuart et al, 1974), stains a median group of cells on the dorsal surface of the thoracic and abdominal ganglia of the cockroach, (Dymond & Evans, 1979). These cells are thought to correspond to the dorsal unpaired median neurones of the locust. In the cockroach

Table: 2 Criteria for identification of a neurotransmitter

1 Presence and metabolism

It should be possible to localise the suspected transmitter - either directly by specific stains, fluorescence or autoradiographic markers, or indirectly by selectively staining the synthesizing and or inactivating enzymes involved in their metabolism.

2 Release

During stimulation of the presynaptic element, the suspected transmitter should be released from the nerve ending in amounts commensurate with its biologically effective concentration. It should be possible to collect this efflux for identification and assay, but this is often difficult in practice due to the inaccessibility of many synaptic junctions.

3 Biological activity

The most important criterion for a suspected transmitter is that it should have the same physiological actions as the natural transmitter released on nerve stimulation. Thus it is essential to establish that exogenous application of the putative transmitter produces identical changes in membrane potential, conductance, permeability, ionic equilibrium potential and type of transient postsynaptic event (e.g. epsp, ipsp, AP) as well as AP rate. Any subsequent alterations that normally follow synaptic action, such as changes in intracellular concentrations of cyclic AMP or cyclic GMP, must also be mimicked.

4 Behaviour towards drug action

It is essential that the behaviour of the suspected transmitter be altered by pharmacological agents in a predictable way. The effect and time-course of agonists should mimic its action while antagonists, re-uptake blockers and potentiators, and other synergists should influence the putative transmitter in an identical fashion.

from Leake and Walker, 1980.

cerebral ganglia intense staining with neutral-red is seen in the mushroom bodies (Corpora pedunculata) in the region of the globuli cell body layer around the neuropile. The Falck-Hillarp technique, which is a fluorescent stain for catecholamines, does not reveal any histofluorescence. This suggests that the amines are not catecholamine in nature, and that the neutral-red staining is due to the presence of a non-catecholamine, possibly octopamine, (Dymond & Evans, 1979). Similar results have been obtained from the metathoracic ganglion of the locust. The dorsal unpaired median neurone, (DUMETi), which innervates the extensor tibia of the locust metathoracic leg, is stained by neutral-red. Radioenzymatic assay of the DUMETi soma shows that octopamine is only detected in regions where there is neutral-red staining, (Evans & O'Shea, 1978). The globuli cells of locust cerebral ganglia are also stained by neutral-red, (Dymond & Evans, 1979), indicating that octopamine is also present in central neurones.

1:4:3:4b Metabolism

1:4:3:4bi Synthesis

The proposed synthetic pathway for octopamine synthesis is the decarboxylation of tyrosine to tyramine and the subsequent β hydroxylation of tyramine to octopamine, (Evans, 1980). The enzyme tyramine β hydroxylase which converts tyramine to octopamine has been isolated from lobster (Homarus americanus), nervous tissue, (Wallace, 1976) which has octopamine containing neurones, (Evans et al, 1976). Tyramine β hydroxylase has been characterized and is similar to mammalian dopamine β hydroxylase, it can convert dopamine to noradrendaline, as well as tyramine to octopamine. The enzyme appears to be localized in the octopamine containing neurones as the enzyme activity can be correlated with the octopamine content of various regions of lobster thoracic ganglion nerve roots, (Wallace, 1974). DUMETi cell clusters from locust thoracic ganglia are able to synthesise radiolabelled octopamine and tyramine when incubated in the presence of [^{3}H] tyrosine, (Hoyle & Barker, 1975). In the DUM somata the predominant labelled metabolite is octopamine, in almost twice the amount of tyramine.

1:4:3:4bii Uptake

In vertebrate nervous system the major mechanism by which the actions of noradrenaline, dopamine and 5-HT at release sites are terminated is uptake, (Iversen, 1973). High affinity uptake mechanisms transfer the released amines back into the presynaptic terminal, termed uptake I, whilst lower affinity uptake mechanisms take up amines into non-neuronal tissue (termed uptake II) where

enzymatic degradation may take place, (Iversen, 1967). Similar mechanisms have been described for the nerve cord of the cockroach, for the uptake of octopamine. Three components of uptake are present; high and low affinity sodium sensitive, and sodium insensitive components. The sodium insensitive component is relatively insensitive to temperature changes, unlike the others. This is suggestive of a diffusive process, although competition experiments reveal a certain degree of specificity, (Evans, 1978b). The high affinity component of octopamine uptake has many parallels with the noradrenaline uptake system into rat heart, (Iversen, 1967). The most potent inhibitor of octopamine uptake into the cockroach nerve cord is tyramine. Structure activity relationship studies show that the uptake system in the cockroach prefers phenolamines whereas that in rat heart prefers catecholamines. Dopamine is less effective than tyramine and adrenaline less effective than synephrine as substrates for uptake, (Evans, 1978 b) whereas the reverse is true in rat heart, (Iversen, 1967). The uptake inhibitor drugs desimipramine and imipramine which inhibit noradrenaline uptake into rat heart also inhibit octopamine uptake into cockroach nerve cord.

1:4:3:4biii N-acetylation

Another route for the inactivation of released amines would be enzymatic degradation. The major enzymatic pathway in the insect nervous system appears to be N-acetylation. The enzyme N-acetyltransferase replaces a hydrogen on the amino group with an acetyl group. The N-acetylation of octopamine is predominant in locust brain and thoracic ganglia. The transferase

activity is enhanced by the addition of acetyl co-enzyme A (Acetyl CoA) and radiolabelled N-acetyl-octopamine is produced when the tissues are incubated with[^{14}C]Acetyl CoA, (Hayashi et al, 1977). No monoamine oxidase (MAO), activity is detected in locust nervous tissue compared to 2.73 n moles/mg protein/min of N-acetyl transferase activity. MAO is the major amine degrading enzyme in mammals, (Kakimoto & Armstrong, 1962) and is inhibited by iproniazed which has no effect on the N-acetyltransferase activity, (Hayashi et al, 1977).

1:4:3:4biv Other Catabolic pathways

Other enzymic pathways for the inactivation of released amines include sulphate conjugation of octopamine and dopamine in the lobster nervous system, (Kennedy, 1978) and β-alanine conjugation of octopamine in lobster and moth nervous systems, (Kennedy, 1977 ; Moore et al, 1978).

1:4:3:4c Release

Octopamine has been shown to be present in localized regions of the second thoracic nerve root of the lobster, (Wallace et al, 1974) in the same regions as tyramine β hydroxylase, (Wallace, 1976). When the nerve roots are incubated in the presence of either [^{3}H]tyrosine or [^{3}H]tyramine radiolabelled octopamine is released, (Evans et al, 1975a). A single pulse of 100 mM potassium (a depolarising concentration) causes a sharp increase in the release of radiolabelled octopamine but successive pulses result in a decreased amount of [^{3}H] octopamine released each time. The application of a long potassium pulse of 30 minutes causes

an initial peak of released octopamine, which declines to a steady plateau and is maintained for the duration of the pulse. The release is calcium-dependent, and exposure to 40 mM cobalt inhibits the potassium induced release. Octopamine is selectively released, no tyrosine is present in the efflux after equilibration, (Evans et al, 1975 b).

1:4:3:4d Pharmacology

Firing of the DUMETi neurone of locust metathoracic ganglion causes a slowing of the intrinsic rhythm of contraction and relaxation of the extensor tibiae muscles. This effect can be mimicked by superfusing the muscle with octopamine, (Evans & O'Shea, 1978). When the DUMETi neurone is stimulated electrically by antidromic stimulation, spike activity in the soma is recorded. A single spike in DUMETi causes a significant lengthening of the period of extensor muscle contractions from 20.8 seconds to 21.8 seconds between contractions. Stimulation at a frequency of 1 Hz results in a decrease of both the frequency and amplitude of contraction. If a 2 minute pulse of 10^{-6}M octopamine is applied, the muscle contractions are abolished, and return 56 seconds after the muscle is washed with saline. The effect of octopamine is dependent on the presence of extracellular calcium, as in the presence of calcium-free saline it fails to abolish the rhythm and the frequency and amplitude become highly irregular. The apparent threshold for the effect of octopamine on the myogenic rhythm is between $10^{-9}$and 10^{-10}M, and it is completely abolished by 8×10^{-7}M. The effect on the myogenic rhythm is found to have a well-defined

structure activity relationship, the most effective compound being synephrine followed by octopamine and phenylethanolamine. They all have the ability to cause a 100% reduction in the frequency of contraction. Other amines are only slightly effective. The octopamine effect is blocked by the α-adrenergic receptor blocking agent phentolamine, but not by the β-receptor blocker propranolol, and is stereospecific for the D(-) isomer of octopamine, (Evans, 1980). This suggests a receptor with a high affinity for octopamine on the muscle. The DUMETi neurone can also have indirect effects on the extensor muscle when it is stimulated at the same time as the motoneurones. DUMETi potentiates the tension generated in the muscle by spike activity of the slow excitatory motoneurones (SETi). It reduces the duration of each twitch contraction by SETi by increasing the rate of muscle relaxation and increases the amplitude of the SETi synaptic potential. These effects can also be mimicked by the application of exogenous octopamine, (O'Shea & Evans, 1979). The site of octopamine action is thought to be pre-synaptic. The presence of presynaptic receptors is revealed by an increase in the frequency of spontaneous miniature end-plate potentials in the presence of octopamine. This effect is blocked by phentolamine but not propranolol. Both the presynaptic and muscle receptors for octopamine possess some of the characteristics of vertebrate α-adrenoceptors. The phenomenon of potentiating neurotransmission at the neuromuscular junction is similar to that observed at the vertebrate neuro-muscular junction. The defatiguing effect of both the application of adrenaline and sympathetic nerve stimulation termed the

"Orbeli phenomenon", (Orbeli, 1923) is mediated by presynaptic α-adrenergic receptors, (Bowman & Nott, 1969).

1:4:3:4e Stimulation of adenylate cyclase

Octopamine has been shown to stimulate adenylate cyclase activity in both intact and homogenized invertebrate nervous tissue. In the intact cockroach throacic ganglion the highest level of accumulated cyclic AMP occurs in the presence of both theophylline(a phosphodiesterase inhibitor) and octopamine rather than in the presence of theophylline or octopamine alone. In homogenates of cockroach thoracic ganglia, adenylate cyclase is maximally stimulated by an octopamine concentration of 10^{-4} M. No effect on endogenous phosphodiesterase activity is observed indicating that the increase in cyclic AMP is due to a stimulation of adenylate cyclase rather than an inhibition of phosphodiesterase activity, (Nathanson & Greengard, 1973). Structure activity studies on the octopamine-stimulated adenylate cyclase of cockroach brain indicate that it is linked to a highly specific receptor. Activity is specific for the naturally occuring D(-) isomer of octopamine which is 200 times more potent than the L(+) isomer. Any changes in the molecule reduce potency, none are found to increase it, the most potent molecule being D(-) octopamine, (Harmar & Horn, 1977). Octopamine (10^{-4} M) also stimulates adenylate cyclase in intact Aplysia abdominal ganglion, (Levitan et al, 1974) and in homogenates of cerebral ganglia from locust, (Kilpatrick et al, 1980).

1:4:4 Summary and possible Physiological role of Octopamine

1:4:4:1 In Mammalian Nervous Tissue

No physiological role has as yet been assigned for octopamine in vertebrates. It is present in various tissues, such as neuronal, heart and kidney, (Kakimoto & Armstrong, 1962). It is associated with noradrenergic neurones in mammalian peripheral and central nervous system and it has been suggested that it may be a false transmitter or a co-transmitter with noradrenaline, (Molinoff & Axelrod, 1972).

1:4:4:2 In Invertebrate Peripheral Nervous System

In invertebrate species octopamine is also associated with nervous tissue and is present in much larger amounts than in mammals. Much evidence has been presented for its role as a neurotransmitter and fulfills all the criteria for the identification of a neurotransmitter as listed by Phillis, (1970), although not all in the same species. It also complies with Burnstock's, (1976) re-definition of a neurotransmitter as being a 'substance that is synthesized and stored in nerves, released during nerve activity and whose interaction with specific receptors in the postsynaptic membrane leads to changes in postsynaptic activity'. Octopamine appears to have the same physiological effects in invertebrates as noradrenaline has in mammals. The metabolic and neuronal actions of exogenous octopamine are similar to the arousal of the animal under stress conditions, and the subsequent activation of a fight or flight syndrome. Octopamine may indeed be the sympathetic transmitter in insects and the presence of very small amounts of noradrenaline may be due to

its erroneous synthesis by the hydroxylation of dopamine, due to accidental uptake from adjacent dopaminergic nerves, (Robertson & Juorio, 1976).

1:4:4:3 In Invertebrate CNS

In many preparations octopamine has also been localized in specific regions of the central nervous system and can stimulate adenylate cyclase in invertebrate brain. Octopamine may also be a central transmitter in invertebrates. As in mammalian nervous systems, studies have concentrated on the peripheral nervous system rather than the central nervous system because of its greater accessibility. New and more specific approaches will have to be employed to ascertain the role of octopamine in insect CNS. A possible approach may be the use of immuno-cytochemical localization of synthetic enzymes and uptake mechanisms, as these have been suggested as being the most useful 'markers' for localizing a putative central neurotransmitter, (Storm-Mathieson, 1977). Micro-iontophoretic application of octopamine and drugs to regions in which octopamine has been localized, i.e. the mushroom bodies of the cerebral ganglia of locust and cockroach, (Evans, 1980) may also provide further evidence for a central neurotransmitter role.

1:5 Cyclic Adenosine 3', 5'-Monophosphate (Cyclic AMP)

1:5:1 History

Sutherland and Rall, (1957) observed that the actions of adrenaline and glucagon on dog liver phosphorylase formation were mediated by a heat-stable factor. The factor which accumulated when liver homogenates were incubated in the presence of ATP and hormones was subsequently isolated and characterized and found to be cyclic adenosine - 3', 5' monophosphoric acid, (Sutherland & Rall, 1958; Lipkin et al, 1959). It was proposed by Sutherland that cyclic AMP may have a general role as a 'secondary messenger'. The first messenger being hormone, drug, or neurotransmitter, acting on receptors at the cell surface, causing an increase in the intracellular level of cyclic AMP, which in turn stimulates a response within the cell.

1:5:2 Localization

The presence of cyclic AMP has been detected in plasma, cerebrospinal fluid, gastric juice, milk and urine in concentrations of 10^{-8} - 10^{-6} M, (Sutherland, 1971). The source of this extracellular cyclic AMP is not known although in urine it is thought that the kidney is a major contributor, (Broadus et al, 1970). Rats daily excrete several times more cyclic AMP than is contained in the whole body and a number of observers have suggested that changes in the extracellular level may reflect pathological states due to hormonal imbalances, (Murad, 1973). Cyclic AMP is physiologically an intracellular compound, it cannot readily enter cells as it is not lipid soluble, it is therefore

synthesized de novo within the cell in response to extracellular hormonal influences.

1:5:3 Metabolism

1:5:3:1 Adenylate Cyclase

1:5:3:1a Distribution

Cyclic AMP is formed from ATP by the enzyme adenylate cyclase (EC 4.6.1.1) in the presence of Mg^{2+} ions, (Rall & Sutherland, 1962) and has been found in every animal tissue so far examined. The enzyme has also been reported in plant tissues, where it is thought to be stimulated by various hormones into synthesizing either 2', 3' or 3', 5' - cyclic AMP which have been shown to participate in various aspects of plant metabolism, (Newton, 1974).

In broken cell preparations adenylate cyclase activity is almost always found associated with the particulate fraction but not the soluble fraction of the tissue homogenate, (Sutherland & Rall, 1958). In a few species of bacteria, adenylate cyclase activity is found in the soluble fraction. The species Brevibacterium liquefaciens, contains a high soluble and low particulate adenylate cyclase activity after homogenization, (Ide, 1971) although in vivo the enzyme may be membrane-bound.

In purified bovine thyroid membranes, cyclic AMP production is stimulated by thyroid stimulating hormone. The adenylate cyclase activity co-purifies with Na^{+} - K^{+} - ATPase, and 5' nucleotidase, which are thought to be valid plasma membrane enzyme markers, (Wolff & Jones, 1971). This is taken as evidence that the cyclase

is membrane bound.

1:5:3:1b Structure and Activation of the Adenylate Cyclase System

The present concept of the adenylate cyclase system is that the catalytic unit is an integral membrane protein occupying sites on the inner surface of the plasma membrane, and the hormone receptors are integral membrane proteins on the outside of the plasma membrane. When the receptor is not occupied the receptor is not coupled to the enzyme, and receptor occupancy leads to the formation of a complex between them, (Kahn, 1976). The β-adrenergic receptor can be separated from the cyclase with the detergent digitonin. It is found that a protein with high specific binding of [^{3}H] dihydroalprenolol (a β-adrenoceptor antagonist) and a protein with high adenylate cyclase activity are eluted in different fractions from Sepharose gels, indicating that the β-adrenergic receptor and the cyclase enzyme are discreet macromolecular entities, (Limbird & Lefkowitz, 1977). More evidence for the separate cyclase/receptor concept, has been provided by studies on the number of receptors, and cyclase:

i) The number of β-adrenergic receptors in rat erythrocytes varies independently of the adenylate cyclase, (Charness et al, 1976).

ii) The β adrenergic receptors of turkey erythrocyte can be coupled to rat adrenal tumour cells not responsive to β-adrenergic stimulation of adenylate cyclase by isoprenaline. Rat adrenal tumour cells contain adenylate cyclase but no β-adrenergic receptors, fusion of receptor

with the tumour cell confers it with the ability to activate adenylate cyclase in response to β-adrenergic receptor stimulation, (Schramm et al, 1977).

iii) In a novel variant of mouse S49 lymphona cells the β-adrenergic receptors are permanently uncoupled from the catalytic unit of adenylate cyclase, (Haga et al, 1977). Isoprenaline does not stimulate adenylate cyclase activity, yet there is a high specific binding of [^{125}I] Iodohydroxybenzyl pindolol a valid measure of β-adrenergic receptor concentration, (Ross et al, 1977).

It has also been shown by the technique of radiation inactivation by an electron beam, that coupling between hormone receptor and cyclase is one to one, and that the cyclase and receptor are mobile as well as independent. Occupancy of the receptor is thought to allow the receptor and cyclase to couple and form a functional complex which spans the membrane, (Hoslay et al, 1977).

Another component of the hormone sensitive adenylate cyclase complex is a guanylnucleotide binding protein (N-protein), which on binding GTP activates the cyclase, (Rodbell, 1980). In the frog erythrocyte the regulatory protein N forms a stable complex with the β-adrenergic receptor in the presence of agonist to form a ternary complex of agonist-receptor-N, which facilitates the binding of guanine nucleotides to N-protein. The resulting N-protein-GTP complex in turn conveys nucleotide-dependent stimulation of adenylate cyclase, (Stadel et al, 1981). It

is thought that as the hormone dissociates from the receptor, activated N-protein-GTP stimulates adenylate cyclase which is deactivated by the hydrolysis of GTP to GDP by N-protein, (Rodbell, 1980). The N-protein acts as a 'shuttle' between receptor and cyclase, (Citri & Schramm, 1980).

1:5:3:2 Phosphodiesterase

Cyclic AMP is broken down by hydrolysis to 5'-AMP. The reaction is catalysed by cyclic-nucleotide phosphodiesterase, (Butcher & Sutherland, 1962). Phosphodiesterase has a tissue and species distribution is similar to that of adenylate cyclase. Phosphodiesterase is usually present in the soluble fraction of cell homogenates although in brain where the highest concentration is found, the phosphodiesterase activity resides in the membrane fraction, (Gaballah & Popoff, 1971; Cheung, 1967). It has been proposed that a low Km cyclic AMP specific phosphodiesterase is membrane bound and a high Km cyclic AMP or GMP phosphodiesterase is soluble, (Thompson & Appleman, 1971). In addition another feature is that Ca^{2+} is able to activate phosphodiesterase. Although only 2 of the 6 phosphodiesterase isoenzymes extracted from rat cerebellum are activated by Ca^{2+}, (Uzunov & Weiss, 1972).

Phosphodiesterase is inhibited by the methyl xanthine class of compounds; caffeine, theobromine, aminophylline and theophylline being the most potent. Papaverine,the isoquinolone opium alkaloid,is also a potent inhibitor of phosphodiesterase especially from smooth muscle. Theophylline and other phospho-

diesterase inhibitors are frequently used in the assay of adenylate cyclase activity to prevent the degradation of cyclic AMP or to potentiate their physiological effects, (Appleman et al, 1973; Robison et al, 1968).

1:5:4 Role in Nervous Tissue

1:5:4:1 Mammalian

The activity of adenylate cyclase is stimulated by an extremely large number of biologically active molecules, producing cyclic AMP in a wide variety of tissues, (Jost & Rickenberg, 1971; Weller, 1979). There is increasing evidence that cyclic AMP may play an important and central role in neurotransmission. The intracellular actions of many putative neurotransmitters seem to be mediated by cyclic AMP and possibly cyclic GMP, (Nathanson, 1977). The criteria proposed for the mediation of neurotransmission by a cyclic nucleotide, (Greengard, 1978) are presented in Table 3.

Electrical stimulation of the preganglionic fibres of the rabbit superior cervical ganglion causes an increase in the intracellular cyclic AMP level. Dopamine is thought to be the ganglionic neurotransmitter which mediates the slow inhibitory post synaptic potential (S-IPSP) illicited by electrical stimulation. Dopamine mimics the electrically stimulated S-IPSP, (Libit & Tosaka, 1970). Dopamine itself increases the level of cyclic AMP in the ganglion as does electrical stimulation, (Kebabian & Greengard, 1971). In the presence of theophylline the S-IPSP is potentiated, suggesting that cyclic AMP mediates the S-IPSP. The dopamine

Table: 3 Some criteria for mediation by a cyclic nucleotide of a postsynaptic permeability change

A Synaptic activation and cyclic nucleotide levels

1 Electrical stimulation of the presynaptic input should increase tissue levels of the cyclic nucleotide.

2 This increase in cyclic nucleotide level should be antagonized by pharmacological agents that block the postsynaptic permeability change.

3 The increase should not occur when transmitter release is prevented with low Ca^{++}/high Mg^{++}.

4 The increase should not occur when the postsynaptic cells are antidromically activated.

B Neurotransmitters and cyclic nucleotide levels

1 Levels of the cyclic nucleotide should increase when the intact tissue (or blocks or slices of the tissue) is exposed to the neurotransmitter thought to be responsible for the postsynaptic permeability change.

2 This increase should be antagonized by agents which block the neurotransmitter-induced postsynaptic permeability change.

C Neurotransmitters and adenylate (or guanylate) cyclase

1 A neurotransmitter-sensitive adenylate (or guanylate see Section 1:6 page 44) cyclase should be demonstrated in cell-free preparations of the tissue.

2 Activation by the neurotransmitter of this neurotransmitter-sensitive adenylate (or guanylate) cyclase should be blocked by the antagonists referred to in sections A2 and B2 of this table.

D Cytochemistry

1 Cytochemical techniques should demonstrate that cyclic nucleotide levels increase specifically in the postsynaptic cells in response to synaptic activation.

2 Similarly, cytochemical techniques should demonstrate that cyclic nucleotide levels increase specifically in the postsynaptic cells in response to the appropriate neurotransmitter.

E Phosphodiesterase inhibitors

1 Phosphodiesterase inhibitors should potentiate the effects of activating the synaptic pathway, and of applying the putative neurotransmitter, on the increase in cyclic nucleotide levels.

2 Phosphodiesterase inhibitors should potentiate the effects of activating the synaptic pathway, and of applying the putative neurotransmitter, on the postsynaptic permeability change.

F Application of cyclic nucleotides

1 The cyclic nucleotide should mimic the physiological effects of activating the synaptic pathway and of applying the putative neurotransmitter.

from Greengard, 1978

induced S-IPSP is potentiated by theophylline and monobutyryl cyclic AMP can itself induce an S-IPSP, (McAfee & Greengard, 1972). This provides direct evidence of a definite role for cyclic AMP in synaptic transmission. Neurotransmitter sensitive adenylate cyclase have been demonstrated in various regions of mammalian brain, (Nathanson, 1977).

1:5:4:2 Invertebrate

It has been shown that octopamine, dopamine and 5-HT all stimulate adenylate cyclase activity in cockroach thoracic ganglia in a concentration-dependent manner. The amount of cyclic AMP formed in response to octopamine, dopamine and 5-HT is additive with any combination added, strongly indicating the presence of separate receptors for each. Octopamine, dopamine and 5-HT sensitive adenylate cyclase have also been identified in other invertebrate preparations, (Nathanson, 1977).

In the isolated abdominal ganglion of Aplysia californicus, previously incubated with [^{3}H] adenine, the synthsis of labelled cyclic AMP doubles after electrical stimulation of the nerves at physiological frequencies. The accumulation of cyclic AMP is dependent on synaptic activity as the inhibition of neuro-transmission by raising the Mg^{2+} ion concentration 4 times greater than normal also inhibits the increase in cyclic AMP levels. The inhibition of cyclic AMP production by Mg^{2+} is not an effect directly on synthesis, as it does not inhibit the elevation of cyclic AMP levels in response to exogenous dopamine or 5-HT, (Cedar et al, 1972; Cedar & Schwartz, 1972).

Octopamine, dopamine and 5-HT stimulate adenylate cyclase in homogenates of moth brain, (Bodnaryk, 1979), Manduca sexta nerve cord, (Taylor & Newburgh, 1978) and Schistocerca gregaria cerebral ganglia, (Kilpatrick et al, 1980). Relatively high concentrations of octopamine and other putative neurotransmitters have no effect on the endogenous phosphodiesterase activity in cockroach brain, (Nathanson, 1976) or Manduca sexta nerve cord when compared to theophylline inhibition, (Taylor & Roberts, 1979). This indicates that the observed increases in cyclic AMP levels are due to a stimulation of adenylate cyclase rather than an inhibition of phosphodiesterase.

Cyclic AMP can act presynaptically. At the cockroach neuromuscular junction, 10^{-4} M cyclic AMP added to the bathing medium, increases the frequency of miniature end-plate potentials (mepp) recorded from the muscle fibres, by a factor of 3. Addition of caffeine, theophylline or ICI 163197 (a non-methyl xanthine phosphodiesterase inhibitor) all have similar effects to cyclic AMP. The increase in mepp frequency is not accompanied by any change in the resting membrane potential which stays constant throughout, (Wareham, 1978).

1:5:5 Cell Location of the increase in cyclic AMP levels in Nervous Tissue

1:5:5:1 Mammalian

The studies on adenylate cyclase activity mentioned previously do not reveal the precise cellular location of the accumulated cyclic AMP. Both mammalian and invertebrate brain are heterogenous with respect to cell type.

Each neurone is surrounded by many supporting glial cells which have been shown to possess adenylate cyclase activity in mammalian brain. Glial tumour cells have the ability to synthesize cyclic AMP in response to the exogenous application of noradrenaline or dopamine, (Schultz et al, 1972). It was proposed by Newburgh & Rosenberg, (1972) that the hormone-sensitive adenylate cyclase is primarily localized in glial cells. In various regions of rat and rabbit brain noradrenaline and dopamine stimulate adenylate cyclase in both neuronal and glial cells, (Palmer, 1973).

1:5:5:2 Invertebrate

Separated neuronal perikarya and glia from the nerve cord of Manduca sexta have been studied for the effects of several putative neurotransmitters on the level of cyclic AMP accumulation. Exposure to 5-HT results in a 33,000-fold increase in cyclic AMP in the neuronal fraction whereas the glia only show a 430-fold increase, approximately 10 times less. Theophylline also increases the level of cyclic AMP in the neuronal fraction but to a 10 times lesser extent. No effect of theophylline is observed in the glial fraction, (Taylor et al, 1976).

It is clear that in view of the contribution of glial cells to neurotransmitter-sensitive adenylate cyclases in mammalian brain, that glia might play an important part in the cyclases of invertebrates. The work of Taylor et al, (1976) indicates that invertebrate glial cells have the ability to synthesize cyclic AMP in response to putative neurotransmitters and that its metabolism and/or control may be different than in neurones.

1:6 Cyclic GMP in Nervous Tissue

Guanylate cyclase the enzyme which catalyses the formation of guanosine 3', 5', cyclic-monophosphate was first studied in a variety of rat tissues, including brain. The formation of cyclic GMP in rat brain is approximately 10-fold less than that of cyclic AMP. In general the tissue levels of cyclic GMP are much smaller than cyclic AMP. The formation of cyclic GMP from GTP is not catalyzed by adenylate cyclase, and there is evidence for 2 separate cyclases, (Hardman & Sutherland, 1969).

Neurotransmitters have been shown to increase levels of cyclic GMP in postsynaptic cells. During pre-ganglionic stimulation of bullfrog sympathetic ganglia the level of cyclic GMP doubles after 2 minutes, (Weight et al, 1974). High magnesium ion concentrations and low calcium inhibit the increase in cyclic GMP. The rise in cyclic GMP in response to nerve stimulation is also blocked by the cholinergic muscarinic antagonist atropine, Weight et al, 1974). Acetylcholine and bethanechol both increase cyclic GMP, but not the nicotinic agonist 1, 1-dimethyl 4-phenylpiperazinium in bovine sympathetic ganglion, (Kebabian et al, 1975), suggesting that occupancy of muscarinic cholinergic receptors activates guanylate cyclase. α-Adrenoceptor activation is not normally associated with adenylate cyclase but can reduce or prevent an increase in cyclic AMP caused by β-agonists, (Haslam & Taylor, 1971). In the rat parotid gland both noradrenaline and phenylephrine, (an α-adrenoceptor agonist) stimulate an increase in the cyclic GMP level which is selectively blocked by phentolamine, (Butcher et al, 1976). As with muscarinic

receptors it appears that α-adrenergic receptors may also be linked to guanylate cyclase.

Characterization of the guanylate cyclase has so far been difficult, and it is as yet far from clear which physiological processes regulate its activity. Some of the difficulty may be attributed to the existence of 2 forms of the cyclase, a soluble, and to a lesser extent, a particulate form in almost all tissues examined, (Nakazawa et al, 1976). The regulation of cyclic GMP synthesis is seemingly without direct analogy to the control of cyclic AMP production. Although certain neurotransmitters rapidly increase the intracellular cyclic GMP concentration, neither the soluble nor particulate forms of guanylate cyclase are stimulated in broken cell preparations, (Goldberg & Haddox, 1977). The elevation of cyclic GMP levels in intact cells may be caused by a neurotransmitter stimulated Ca^{2+} influx, as agents which stimulate the production of cyclic GMP also facilitate the entry of Ca^{2+}. The extracellular presence of Ca^{2+} is necessary for the increase in cyclic GMP levels to occur, (Schultz et al, 1973). It is possible that cyclic GMP is involved in regulating Ca^{2+} metabolism, as Ca^{2+} in most cases seems to be responsible for both the physiological response, and the rise in cyclic GMP, (Rasmussen et al, 1975; Berridge, 1975). In muscle fibres of the giant barnacle, a crustacean, the rise in cyclic GMP levels after potassium depolarisation and nerve stimulation are accompanied by the entry of Ca^{2+}. Pre-incubation of the muscles in Ca^{2+}-free saline abolishes the rise in cyclic GMP, (Beam et al, 1977).

1:7 Intracellular Calcium

1:7:1 Physiological Role

1:7:1:1 General

Calcium plays a specific major role in cellular regulation. It is involved in a wide variety of excitation-response coupling processes and in many of these Ca^{2+} acts as an intracellular second messenger. It is involved in the following processes:

1 Contraction in all forms of muscle

2 Secretion

A Exocrine

B Endocrine

C Neurotransmitter release

D Lysosomal enzyme release

E Histamine release from mast cells

3 Photoreceptor activation in rods

4 Mitosis or cell division

5 Fertilization

6 Metabolism

A Thermogenesis

B Gluconeogenesis

C Glycogenolysis

D Glucose transport

E α-ketoglutarate transport

F Steriod and Sterol biosynthesis

G Cyclic Nucleotide metabolism

(Rasmussen & Goodman, 1977).

In this respect though, it does not always act as a second messenger. In the contraction of smooth muscle and the release of neurotransmitter some of the calcium enters from outside the cell, (Di Polo & Beaugé, 1980 ; James-Kracke & Roufogalis, 1980).

1:7:1:2 Nervous Tissue

It has been generally accepted for some time that calcium entry is the major trigger for initiation of neurotransmitter release. Calcium ions injected into the presynaptic nerve terminal of the squid giant synapse evoke transmitter release. Extracellular manganese and magnesium which can block neurotransmission do not prevent the release of transmitter in response to injected calcium, (Miledi, 1973).

Calcium plays a pivotal role in the regulation of cyclic nucleotide metabolism, and appears to control adenylate cyclase in a biphasic manner. Low Ca^{2+} concentrations stimulate, while higher concentrations inhibit activity. Bovine brain adenylate cyclase is inhibited by EGTA, a powerful calcium chelator. The inhibition is reversible by the addition of a free Ca^{2+} ion concentration of 2 μM. Increasing the free Ca^{2+} ion

concentration to 72 μM markedly inhibits the cyclase, (McDonald, 1975).

The phosphodiesterase activity of rat brain is also regulated by Ca^{2+}. The low Km phosphodiesterase activity is greatly stimulated by Ca^{2+} with a maximum effect at a concentration of 10 mM, (Kakiuchi, et al, 1971).

It has been proposed that the influx of Ca^{2+} from extracellular sources, or the release of membrane-bound Ca^{2+} in response to stimuli, activates adenylate cyclase, releasing cyclic AMP into the cytosol. When the Ca^{2+} subsequently arrives at the cell cytoplasm, the soluble phosphodiesterase is activated, returning the cyclic AMP level to basal. This mechanism would allow transient increases in cyclic AMP levels, (Lynch et al, 1976).

1:7:2 Calmodulin

The actions of Ca^{2+} as an intracellular second messenger are mediated through a class of Ca^{2+}-binding proteins of which calmodulin (formerly termed the calcium-dependent regulator, CDR) is the most common. It is a heat stable, acidic polypeptide with a molecular weight of 16,700 daltons. It has no intrinsic enzyme activity of its own though it can regulate a wide variety of enzymic and many other cellular processes.

The complete amino acid sequence of bovine brain calmodulin has been elucidated. It contains 148 amino acids, and commences with an acetylated alanyl residue at the N-terminus. The linear

sequence has 4 regions where the amino acids form a helix-loop-helix calcium binding structure, conferring on it the ability to bind 4 calcium ions, (Watterson et al, 1980).

Binding of calcium to calmodulin induces a conformational change which allows it to interact with receptor proteins. The action of calmodulin on phosphodiesterase has been extensively studied, and in rat cerebral cortex the enzyme is stimulated by Ca^{2+} in the presence of calmodulin. The calcium-calmodulin complex binds to the apoenzyme and activates it. When the free Ca^{2+} ion concentration is reduced by the addition of EGTA, the Ca^{2+}-calmodulin complex dissociates and returns the phosphodiesterase activity to a basal level, (Teshima & Kakiuchi, 1974).

The activation of adenylate cyclase from rat brain membrane fragments by Ca^{2+}/calmodulin is analogous to that of phosphodiesterase, (Lynch et al, 1976). The following criteria for the mediation of a cellular response by calmodulin have been proposed by Cheung, (1980):

1 The presence of sufficient calmodulin.

2 Depletion of endogenous calmodulin should alter the rate of the reaction which should be restored by the addition of exogenous calmodulin.

3 Sequestration of Ca^{2+} with chelators such as EGTA should reduce the calmodulin stimulated activity to basal levels.

4 The inactivation of calmodulin by drugs such as trifluoperazine,

should also reduce the calmodulin-stimulated activity to basal levels.

5 Anticalmodulin antibody should inhibit the calmodulin-dependent process providing that the antibody has access to the calmodulin.

1:8 Protein Kinases

1:8:1 Cyclic AMP-dependent

1:8:1:1 Distribution

The mechanism by which cyclic AMP mediates various cellular processes is by the activation of a single class of enzymes, the protein kinases. The mechanism was first discovered in rabbit skeletal muscle, where cyclic AMP regulates glycogen breakdown. A protein kinase which markedly increased the rate of cyclic AMP-dependent activation of phosphorylase kinase phosphorylation was purified from the muscle, (Walsh et al, 1968). Since then the mechanism has been extended to other systems, neurotransmission in particular.

Cyclic AMP-dependent protein kinases are present in every mammalian tissue examined, (Kuo & Greengard, 1969) and tissues from every phylum of the animal kingdom (Table 4).

1:8:1:2 Specificity

Protein kinases are enzymes which, by definition, catalyse the transfer of a terminal phosphate group from a nucleotide, usually ATP, to an acceptor protein. The specificity of the response to cyclic AMP resides in the nature of the protein kinases and the nature of the acceptor protein. Many protein kinases

Table: 4 Cyclic AMP-dependent protein kinase activity in species from 9 animal phyla

Phylum	Common name (genus)	Protein kinase activity	
		-Cyclic AMP	+Cyclic AMP
Protozoa	Paramecium (Paramecium)[a]	0.28	0.58
Porifera	Sponge (Haliclona)[a]	0.15	0.30
Coelenterata	Jellyfish (Cyanea)[a]	3.72	5.15
Nematoda	Roundworm (Golfingia)[a]	2.10	3.18
Annelida	Sandworm (Nereis)[a]	0.76	1.74
Mollusca			
(class Pelecypoda)	Clam (Mya)[a]	0.87	2.13
(class Cephalopoda)	Squid (Loligo)[b]	3.45	6.01
Arthropoda	Lobster (Homarus)[b]	0.54	6.72
Echinodermata	Starfish (Asterias)[a]	0.29	0.74
Chordata	Fish (Cyprinus)[b]	0.55	1.15

The whole animal[a] or muscle tissue[b] was used to measure intrinsic protein kinase activity.

From Greengard, (1978).

have been found and many have a broad specificity. Examples of specific protein kinases are opsin kinase, which phosphorylates rhodopsin in the retina, and light chain myosin kinase which catalyses the phosphorylation of light chain myosin, (Weller, 1979). Not all protein kinases are cyclic nucleotide-dependent. There are some which catalyse the phosphorylation of phosvitin and other proteins, but not histones, and there are those which phosphorylate histones and other proteins but not phosvitin, (Weller, 1979).

1:8:1:3 Structure and Mechanism of Action

Protein kinases from various tissues appear to have a tetrameric structure. They are composed of 2 regulatory units which are inhibitory to activity, and 2 catalytic subunits, (Rubin & Rosen, 1975). In rat, guinea-pig, and bovine brain there are 2 specific cyclic AMP receptor proteins which appear to be the regulatory subunits of cyclic AMP-dependent protein kinases, (Walter et al, 1978). The 2 types of protein kinase have been designated type I and type II, (Walter et al, 1977). Nervous tissue contains much higher concentrations of the type II than of the type I protein kinase in both the cytosol and synaptic membrane fractions, (Walter et al, 1978).

Type II protein kinases undergo autophosphorylation whereas type I do not. In the absence of cyclic AMP, the regulatory and catalytic subunit of type II kinases are combined, which keeps them in an inactive state. In the inactive state the catalytic subunit phosphorylates the regulatory subunit in an

intramolecular reaction. In the presence of cyclic AMP the regulatory and catalytic subunits dissociate allowing the catalytic subunit to phosphorylate the acceptor protein, (Greengard, 1978).

Phosphorylation of the regulatory unit does not alter cyclic AMP binding, nor is it cyclic AMP-dependent, but it apparently facilitates the dissociation of regulatory and catalytic subunits in the presence of cyclic AMP, (Rosen & Erlichman, 1975). It has been shown that phosphorylation retards the re-association of the regulatory and catalytic units and even in the absence of cyclic AMP the dephosphorylated regulatory units re-combine much faster. It seems that autophosphorylation of type II kinases regulates the proportion of active and inactive kinase, (Rangel-Aldao & Rosen, 1976).

Cyclic AMP-dependent protein kinase highly purified from pig brain, has a molecular weight of 120,000. This dissociates to a catalytic unit of molecular weight 40,000 and a regulatory unit of 90,000, (Severin et al, 1976). The circular dichroism maximum of the regulatory subunit is 215 nm, which decreases on adding cyclic AMP, this is not observed for the catalytic unit. This is similar to a typical enzyme conformational change on binding an allosteric regulator, (Severin et al, 1976).

The protein kinase from bovine brain is specific with respect to nucleotide triphosphate. It will only transfer the γ-phosphate group of ATP to the acceptor protein. Very little phosphate is transferred from GTP, UTP, CTP, dTTP or dATP in

the presence of cyclic AMP. Magnesium ion is necessary for activation by cyclic AMP and other divalent cations such as cobalt and manganese can substitute for magnesium in stimulating kinase activity, although Ca^{2+} at 10 mM causes a 66% inhibition, (Miyamoto *et al*, 1969).

Almost all the actions of cyclic AMP are mediated via cyclic AMP-dependent protein kinase and subsequent phosphorylation of target proteins. There is only one documented exception. Catabolite repression by cyclic AMP in bacteria seems not to involve phosphorylation. Cyclic AMP regulates *E. coli* lac messenger RNA synthesis. Cyclic AMP is required for β-galactosidase and other inducible enzymes, (DeCrombugghe *et al*, 1969). Cyclic AMP stimulates lac messenger RNA synthesis via a direct action on the lac repressor, both *in vitro* and *in vivo*, but there is no evidence that this involves phosphorylation, (Varmus *et al*, 1970).

1:8:2 Cyclic GMP-dependent

Many invertebrate and mammalian tissues contain cyclic GMP-dependent protein kinases in addition to cyclic AMP-dependent kinases, although at considerably lower levels, (Kuo *et al*, 1971).

A cyclic GMP-dependent protein kinase isolated from lobster brain has a Km of 0.075 μM for cyclic GMP, and 3.6 μM for cyclic AMP. This is in contrast to a cyclic AMP-dependent protein kinase isolated from the same tissue which has a Km

of 1.2 μM for cyclic GMP and 0.018 μM for cyclic AMP, (Kuo & Greengard, 1970). This suggests there are 2 separate and specific protein kinases. These may be distinct from one another in their biochemical and physiological actions, (Kuo et al, 1971). Cyclic GMP-dependent protein kinases from arthropods have been found to be more abundant than in vertebrates, and resemble the mammalian cyclic AMP-dependent enzymes.

Cyclic GMP-dependent protein kinase from lobster muscle and other sources are also composed of catalytic and regulatory subunits (Miyamoto et al, 1970). The kinase from bovine lung undergoes autophosphorylation in the presence of Mg^{2+} analogous to the type II cyclic AMP-dependent kinases, (De Jonge & Rosen,1977).

1:8:3 Calcium-dependent

Brain membranes contain a Ca^{2+}-dependent protein phosphorylating system. The Ca^{2+}-dependent protein kinase requires calmodulin as purification of the synaptic membranes results in a loss of Ca^{2+} stimulated phosphorylation. This can be recovered by the addition of boiled cytosol, (calmodulin is soluble and heat stable) or purified calmodulin, (Schulman & Greengard, 1978 a) The Ca^{2+}/calmodulin-dependent protein phosphorylating system in membranes from a variety of tissues is sensitive to low concentrations of Ca^{2+} and maximally stimulates protein phosphorylation at a free Ca^{2+} ion concentration of 10 μM. The system would be responsive to physiological changes in Ca^{2+} ion flux, (Schulman & Greengard, 1978 b). The molecular mechanism of action is still obscure, but it has been suggested

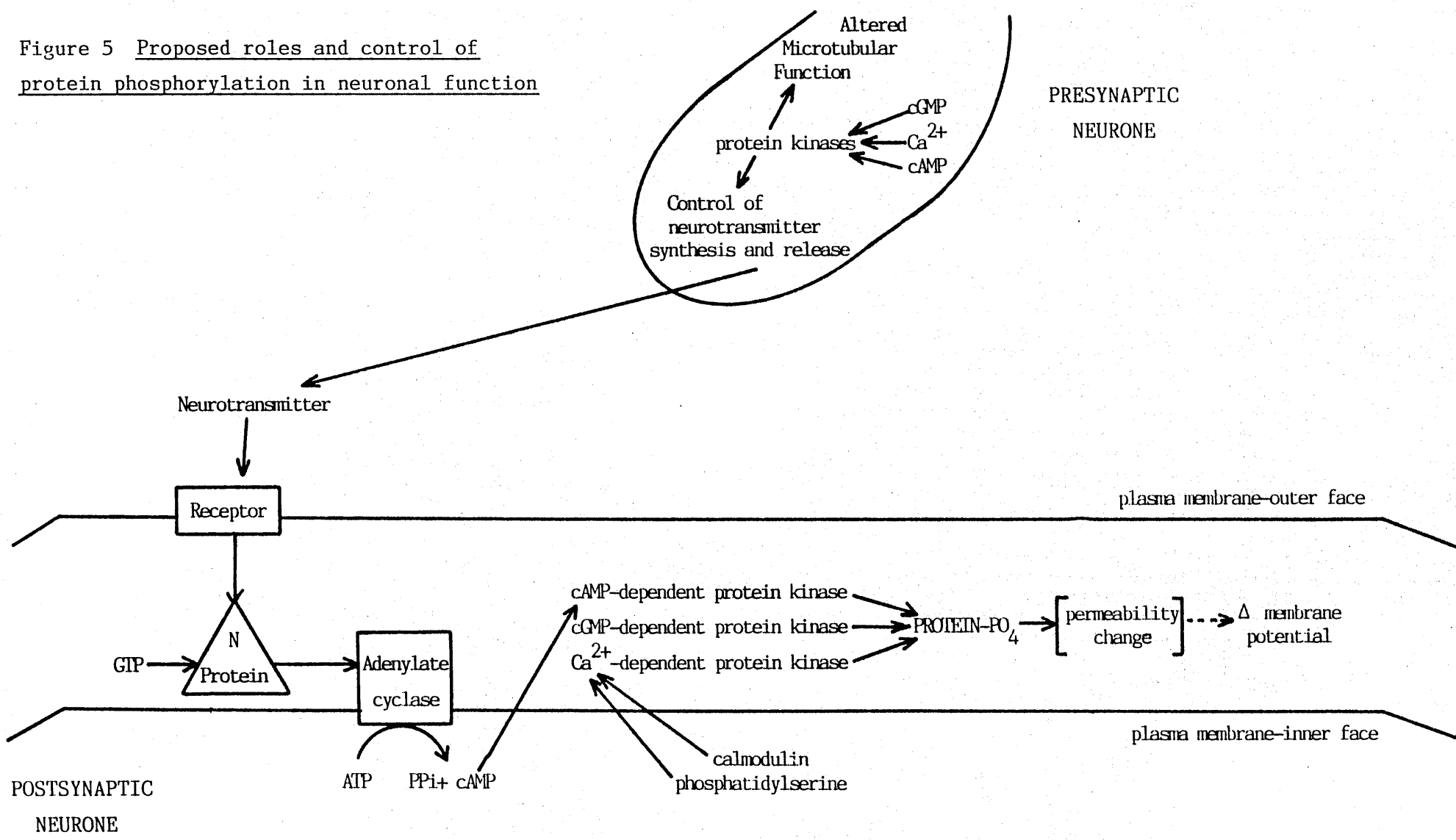
that it is similar to cyclic nucleotide-dependent protein kinase, except that calmodulin may serve as an integral subunit not just an obligatory co-factor for activation, (Wrenn et al, 1980). The calcium-calmodulin complex appears to require free hydrophobic regions for activity. It is inhibited in the presence of Ca^{2+} in a competitive fashion by 2-p-toluidinylnapthalene-6-sulphonate which is known to interact with hydrophobic portions of proteins. It has been proposed that the binding of calcium to calmodulin induces a conformational change which exposes hydrophobic groups, which may be necessary to activate calmodulin-dependent kinases, (Tanaka & Hidaka, 1980). There are at least 4 different types of Ca^{2+}/calmodulin-dependent protein kinases in brain, (Cohen, 1982 ; Nestler & Greengard, 1983).

In addition to calmodulin mediated Ca^{2+}-dependent phosphorylation, a phospholipid Ca^{2+}-dependent phosphorylation has been described. Ca^{2+}/phosphatidylserine stimulates protein phosphorylation in the cytosol of rat and guinea-pig cerebral cortex, where Ca^{2+}/ calmodulin has a minimal effect. Both calmodulin and phosphatidylserine are equally effective in stimulating Ca^{2+}-dependent phosphorylation in a total particulate fraction, (Wrenn et al, 1980). This new component of Ca^{2+}-dependent phosphorylation presumably functions in a complementary manner with calmodulin.

1:9 Protein Phosphorylation

Specific protein kinases stimulated by diverse regulatory agents such as cyclic AMP, cyclic GMP and Ca^{2+}, have a final common pathway which results in protein phosphorylation (Figure 5)

Figure 5 Proposed roles and control of protein phosphorylation in neuronal function



Adapted from Greengard, 1978 ; Nestler & Greengard, 1983.

which may activate enzymes and alter structural proteins, (Weller, 1979).

1:9:1 Evidence for the involvement of protein phosphorylation during synaptic activation

The application of electrical pulses to respiring guinea-pig cerebral cortical slices promotes the net phosphorylation of protein in a membrane fraction, (Trevor & Rodnight, 1965), under conditions which result in the release of catecholamines and other neurotransmitters, (Katz & Chase, 1970). Tetrodotoxin which is known to block impulse conduction in nerve axons by preventing depolarisation induced Na^+ influx, (McIlwain et al, 1969), also prevents the increase in protein phosphorylation and the increase in cyclic AMP levels by electrical pulses. Tetrodotoxin does not, however, prevent the increase in cyclic AMP and protein phsophorylation in response to putative neurotransmitters, (Reddington et al, 1973).

1:9:2 Function of protein phosphorylation in Neurotransmission

There are at least 4 roles in which cyclic nucleotide-dependent protein phosphorylation is involved in nervous tissue:

1 Neurotransmitter synthesis

2 Cytoskeletal protein function

3 Neurotransmitter release

4 Postsynaptic permeability

Tyrosine hydroxylase which catalyses the conversion of tyrosine

to dopa, is the rate limiting step in the biosynthetic pathway for noradrenaline synthesis. Tyrosine hydroxylase, therefore, regulates the synthesis of noradrenaline and its immediate precursor dopamine, (Lovenberg & Bruckwick, 1975 ; Roth et al, 1975). Cyclic AMP-dependent phosphorylation of tyrosine hydroxylase increases its catalytic activity, (Haycock et al, 1982).

Microtubules have an important role in the maintenance and development of cell shape. Purified microtubules from chick brain contain an associated protein which is phosphorylated by a cyclic AMP-dependent protein kinase, (Sloboda et al, 1975). Neurofilaments are found in axons in close proximity to microtubules and are associated with tubulin in axonal transport, (Hoffman & Lasek, 1975). In bovine brain there are 2 components of neurofilament protein phosphorylation, a cyclic AMP-independent, and dependent component of which the latter is associated with microtubular associated protein, (Yamauchi & Fujisawa, 1983).

Synapsin 1 (formerly termed protein 1) is a phosphoprotein present in particulate synaptic fractions from brain. It is a substrate for cyclic AMP, cyclic GMP and calcium/calmodulin-dependent protein kinases. It is only present in neurones and is associated with synaptic vesicles.It is located at the cytoplasmic surface of vesicles in all nerve terminals so far investigated, (Nestler & Greengard, 1983).

Synapsin 1 is phosphorylated by both cyclic AMP and calcium-

dependent protein kinases at different sites, (Huttner et al, 1981). Neurotransmitters acting through cyclic AMP and depolarizing procedures, (both electrical stimulation and high K^+ acting through calcium) increase the incorporation of phosphate into Synapsin 1, (Nestler & Greengard, 1982). Intraneuronal injection of pure catalytic subunit of cyclic AMP-dependent protein kinase mimics the electrophysiological response to synaptic stimulation and facilitates transmitter release, (Castelluci et al, 1980). This has led to the hypothesis that Synapsin 1 is in some way involved in the process of neurotransmitter release, (Nestler & Greengard, 1982).

In the process of neurotransmission, transmitters released from the terminals of the presynaptic neurone interact with specific receptors located on the outer membrane of the postsynaptic neurone altering its permeability properties. The molecular nature of the premeability change is not yet clearly understood, but there appear to be 2 main possibilities:

A) The receptor-ionophore model; where the receptor is in some way directly coupled to an ionophore in such a manner, that binding of the transmitter to its receptor induces a conformational change in the ionophore leading to alteration of permeability, without the involvement of any enzymes. The nicotinic cholinergic receptor is thought to function in this way, (Popot et al, 1976).

B) The receptor-second messenger model proposed by Greengard, (1976) to explain the function of cyclic nucleotides in

neurotransmission in the light of present evidence. The binding of neurotransmitter to its receptor results in a conformational change which activates adenylate cyclase, allowing it to catalyze the conversion of ATP to cyclic AMP which then stimulates a protein kinase which catalyzes the transfer of a phosphate group from ATP to a protein acceptor. This protein may be a constituent of the plasma membrane ion channel, changing its permeability properties when in a phosphorylated state (Figure 5).

The exact function of membrane protein phosphorylation is not known, but ultimately in neurotransmission there would have to be an ion permeability change in order to generate a membrane potential. The catalytic subunit of cyclic AMP-dependent protein kinase purified from bovine heart can catalyze the phosphorylation of Helix and Aplysia nervous tissue proteins in vitro. Perfusion of intact neurones from Helix with catalytic subunit increases Ca^{2+}-activated K^+ conductance, i.e. increases the K^+ permeability. The increase in permeability is not observed in the presence of catalytic subunit inactivated by 5, 5'-dithiobis (2-nitro-benzoic acid), (De Peyer et al, 1982).

Changes in the phosphorylation of specific membrane proteins have been implicated in the control of ionic permeability in non-neuronal systems also; in toad-bladder, antidiuretic hormone and monobutyryl cyclic AMP decrease the phosphorylation of a specific membrane protein. The decrease in phosphorylation caused by these agents preceeds the change in electrical potential

difference in response to these same compounds, (De Lorenzo et al, 1973 ; Walton et al, 1975). Control of permeability by protein phosphorylation has also been suggested in avian erythrocyte, (Rudolph & Greengard, 1974) and cardiac sarcoplasmic reticulum, (Tada et al, 1975).

1:9:3 Nature of the Protein-bound Phosphate

In cerebral proteins, phosphate has been found to be covalently-bound to the hydroxyl groups of serine and threonine in an ester linkage, and to the γ-carboxyl groups of acidic amino acids such as glutamate and aspartate in an acyl linkage, (Rodnight, 1971). Proteins from liver have been found to contain N-phosphoryllysine and N-phosphorylhistidine, (Walinder et al, 1968) and recently phosphate has been reported to be bound to tyrosine, (Martensen, 1982), but these do not appear to be present in brain.

In cerebral proteins the acyl phosphate is thought to be intimately involved in the mechanism of cation transport and ATP hyrolysis, as it occurs in protein fractions rich in Na^+/K^+-stimulated ATPase, (Kanazawa et al, 1967). The acyl phosphate is thought to be the covalent intermediate in the action of the ATPase, (Nagano et al, 1967), and is relatively labile. The bound phosphate is liberated from protein by mild acid at 40 ^{o}C and follows first order kinetics indicating that it is bound as a single species, (Nagano et al, 1965).

Of the 2 phosphoamino acids containing phosphate via ester linkages the most common is phosphoserine, with only trace amounts of phosphothreonine in cerebral proteins, (Rodnight, 1971). It is not known whether both are present in the same molecule, however, metabolically both appear to behave in the same manner. Phosphoserine is relatively stable in acid but the phosphate can be released by alkaline hydrolysis. This

has been known for many years. Plimmer and Bayliss, (1906) reported that alkali released phosphate from phosphvitin and casein, the 2 classical phosphoproteins from egg and milk. It was later found that alkali treatment of denatured proteins from mammalian tissues also yielded inorganic phosphate, (Schmidt & Thannhauser, 1945 ; Schneider, 1945). This has since been employed as a quantitative measure of phosphoprotein although it appears that a measure of the phosphoserine level after partial acid hydrolysis of the protein is also a valid measure of phosphoprotein, (Rodnight, 1971).

1:10 Protein Phosphatases

1:10:1 Properties and Distribution

The dephosphorylation of phosphoproteins is catalyzed by protein phosphatases of which there are 2 main types, acid and alkaline.

1:10:1:1 Acid Phosphatases

Acid phosphatases, which have a pH optimum in the range 3.0 to 6.0 are composed of between 3 and 5 isoenzymes depending on the tissue and species it is isolated from, (Weller, 1979). They can dephosphorylate a large number of diverse phosphomonoesters in addition to phosphoproteins, but can exhibit a slight degree of specificity depending on their source, (Hollander, 1971). They have a wide distribution and are found in most tissues and species.

Acid phosphatase from rat cerebral cortex is evenly distributed between the mitochondrial (containing synaptosomes), microsomal, and soluble fractions, (Hiroshige et al, 1966).

1:10:1:2 Alkaline Phosphatases

Alkaline phosphatases are dimers which have a pH optimum between 8.0 and 10.0, and require a divalent cation for activity, Mg^{2+} being the most effective for the phosphatase from bovine brain, (Cathala & Brunel, 1973). Their substrate specificity is very broad and they can catalyze the dephosphorylation of almost any phosphomonoester from p-nitrophenyl phosphate to phosphoserine and phosphothreonine from phosphoproteins, (Reid & Wilson, 1971).

Alkaline phosphatase is present in most animal species, as well as bacteria and plants. It is generally concentrated in the cytosol although it is present in membranes from lymphocytes, liver microsomes and kidney tubules, (Weller, 1979).

1:10:2 Mechanism of Action

Both acid and alkaline phosphatases are thought to act via a phosphorylated enzyme intermediate, where the enzyme and phosphate-ester form a noncovalently linked complex. The phosphate then becomes covalently linked to the enzyme and the hydrolysed substrate is released. This is followed by release of the phosphate from the enzyme, (Block & Schlesinger, 1973 ; Weller, 1979).

1:11 Aim of the Project

Octopamine has been shown to be present in nervous tissue from both *Aplysia californica* and *Schistocerca gregaria*, (Robertson & Juorio, 1976 ; Evans, 1980). Incubation of *Aplysia* abdominal ganglia with octopamine results in a marked increase in the cyclic AMP level in the tissue, (Levitan *et al*, 1974). Octopamine stimulates the incorporation of phosphate into specific proteins extracted from the ganglia, which is mimicked by dibutyryl cyclic AMP, (Levitan & Barondes, 1974). Cyclic AMP stimulates the incorporation of radioactive phosphate into protein from homogenates of *Helix* nervous tissue in the presence of $[\gamma^{32}P]$ ATP, (Bandle & Levitan, 1977).

Cyclic AMP levels are greatly increased by octopamine in cerebral ganglia homogenates from *Schistocerca gregaria*, (Kilpatrick *et al*, 1980). In view of the above evidence the aim of the present project was to investigate whether octopamine, cyclic AMP and other agents such as Ca^{2+} or cyclic GMP (which have been shown to affect protein phosphorylation in other systems) have any effect on protein phosphorylation in *Schistocerca gregaria* nervous tissue.

2 MATERIALS AND METHODS

2:1 Chemicals and Materials

Analar reagents were used throughout, fine chemicals were obtained from the following sources:

Adenosine-5'-triphosphate (ATP)	Sigma London Chemical Co., Ltd., Poole, Dorset, U.K.
Adenosine -5'-diphosphate (ADP)	"
Adenosine-5'-monophosphate (AMP)	"
DL-Octopamine hydrochloride	"
Ribonuclease A	"
Bovine Serum Albumin Fraction V (BSA)	"
2-amino-2-hydroxymethylpropane-1,3-diol (Tris)	"
1,3 dimethylxanthine (Theophylline)	"
Sodium lauryl sulphate (SDS)	"
Pronase	"
N^6,O^2-dibutyryladenosine 3':5'-cyclic monophosphate (di butyryl cyclic AMP)	"
Adenosine 3', 5'-monophosphate (cyclic AMP)	Boehringer Mannheim, GmbH Biochimica, West Germany
Guanosine-5'-triphosphate (GTP)	"
Guanosine-5'-diphosphate (GDP)	"
Guanylyl-5'-imidodiphosphate (GppNHp)	"

Ethylene glycol-bis (β aminoethylether) N,N,N',N', tetra acetic acid (EGTA)	BDH Chemicals Ltd., Poole, Dorset, U.K.
Bromophenol Blue	"
Coomassie Brilliant Blue R250	"
Penicillin G	"
Acrylamide	"
N,N'-Methylenebisacrylamide	"
Cyclic AMP Binding Protein	"
Streptomycin	Glaxo Laboratories Ltd., Greenford, U.K.
2-mercaptoethanol	Koch-Light Laboratories, Colnbrook, Bucks, U.K.
2,5,-diphenyloxazole (PPO)	"
Folin-Ciocalteau reagent	Fisons Co., Ltd., Loughborough, Leicester
Triton X-100	Rohm and Haas, U.K. Ltd.
N,N,N',N'-tetramethylethylenediamine (TEMED)	Bio Rad Laboratories Ltd., California, USA
Molecular-Weight Standards	Pharmacia Fine Chemicals A Uppsala, Sweden
Guanosine 3', 5'-monophosphate (cyclic GMP)	Gift from Dr D.P. Leader, Department of Biochemistry University of Glasgow
Calcium-dependent regulator (calmodulin)	"
Protein kinase inhibitor	"

Radiochemicals

[^{32}P] inorganic phosphate, carrier-free	Amersham International PLC, Amersham, Bucks, U.K.
[γ^{32}P] ATP	"

Materials

Eppendorf plastic vials (1.5 ml)	Netheler & Hinz, GmbH Hamburg, West Germany
Insert mini scintillation vials	Sterlin Ltd., Middlesex, U.K.
Polyethyleneimine (PEI) cellulose Cel 300	Macherey-Nagal, Duren, West Germany

Electron Microscopy

Electron micrographs were prepared by Ian Montgomery, Electron Microscopy Unit, Department of Physiology, University of Glasgow.

Table:5 Osmolarity of Solutions

Solution	Section[1]	Osmolarity[2] m Osmols/litre
Homogenizing buffer 6 mM-Tris/maleate	2:5:1:1	58
Homogenizing buffer 0.25 M-sucrose, 100 mM-Tris/HCl	2:5:1:2	437
13% Ficoll/100 mM-Tris/HCl	2:5:1:2	482
Assay buffer 80 mM-Tris/maleate	2:5:2	66
Iso-osmolar saline	2:2	421
S. gregaria haemolymph	-	432

1 The full description of each solution is contained in the relevant section.

2 Measurements were carried out by Ian Montgomery, Electron Microscopy Unit, Department of Physiology, University of Glasgow.

2:2 Tissue Preparation

The adult male and female locusts used in this study were supplied by Larujon Locust Suppliers, c/o Welsh Mountain Zoo, Colwyn Bay, North Wales.

Dissections were carried out with the aid of a binocular microscope. Cerebral ganglia were removed by cutting the cuticle along the mid-line of the head, then laterally to the mouth using fine scissors, making sure that they were always pointing upwards to avoid damaging the underlying tissue. One half of the head cuticle was removed by gently prising it up by the antenna and cutting off the underlying tissue as close as possible to the inner surface of the cuticle. This exposed the cerebral ganglion, which was removed by gently lifting it with fine forceps and cutting it from the other half of the head.

Immediately after dissection, ganglia were placed in a cavity slide containing ice-cold iso-osmolar saline, consisting of; 148 mM-NaCl, 10 mM-KCl, 2 mM-$CaCl_2$, 10 mM-glucose, 100 mM-sucrose, and 10 mM-Tris/HCl, pH 6.8. All adhering connective tissue was then removed and the ganglia were either incubated intact or homogenized within 5 minutes.

2:3 Protein Phosphorylation in Intact Ganglia

2:3:1 Incubation

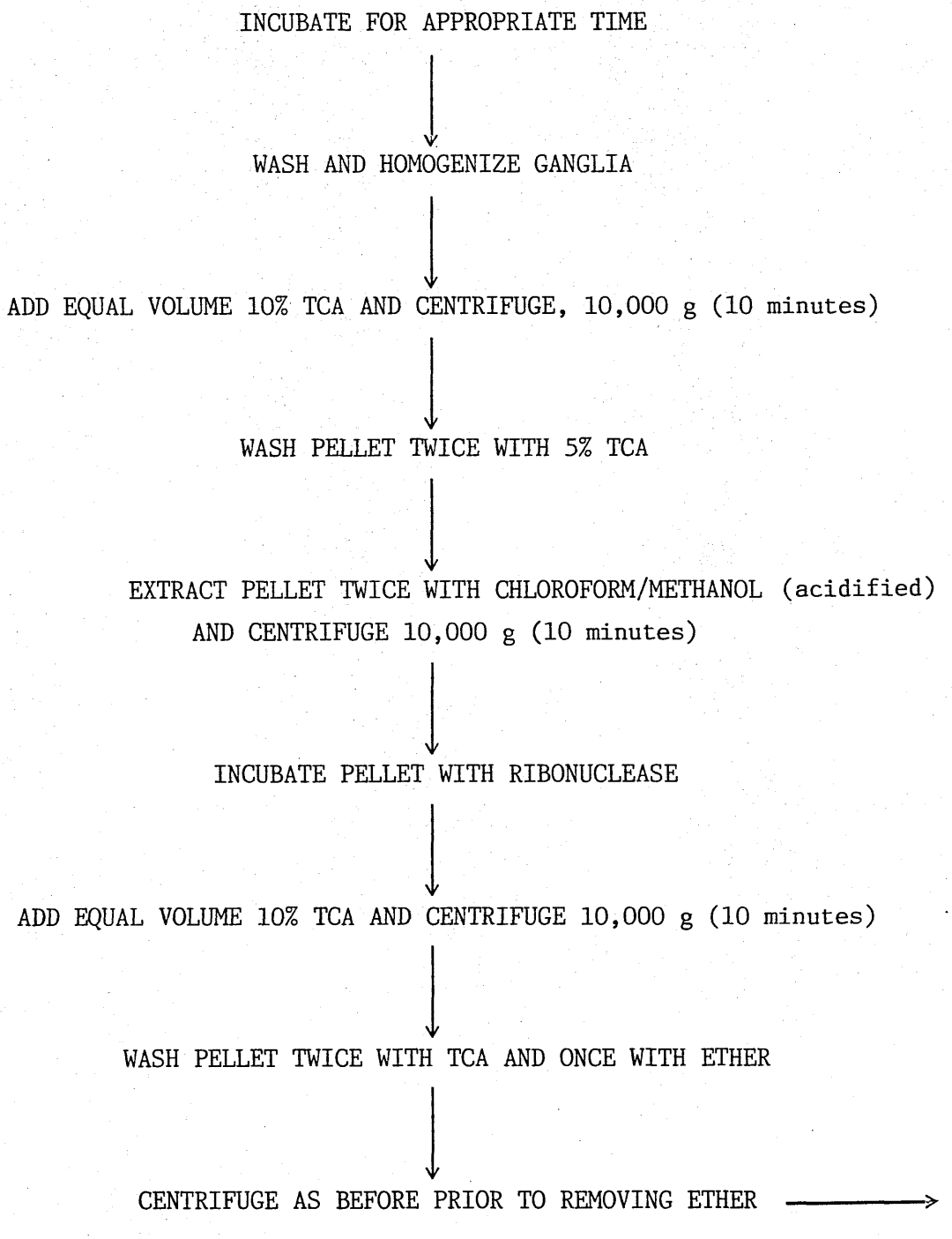
Ganglia were pre-incubated for 5 minutes in Eppendorf vials, (4/vial), containing 480 µl of iso-osmolar saline, (as described in section 2:2, but containing 100 units/ml of Streptomycin, and Penicillin) with 3-5 µCi of ^{32}Pi. Then, either 20 µl of iso-osmolar saline (controls), or 20 µl of, 2.5×10^{-3} M octopamine or other test substance made up in iso-osmolar saline was added to the vials, and incubated* at 30 °C for the appropriate times indicated in the results section.

2:3:2 Protein Pellet Extraction and Determination of Radioactivity (summarized in Figure 6)

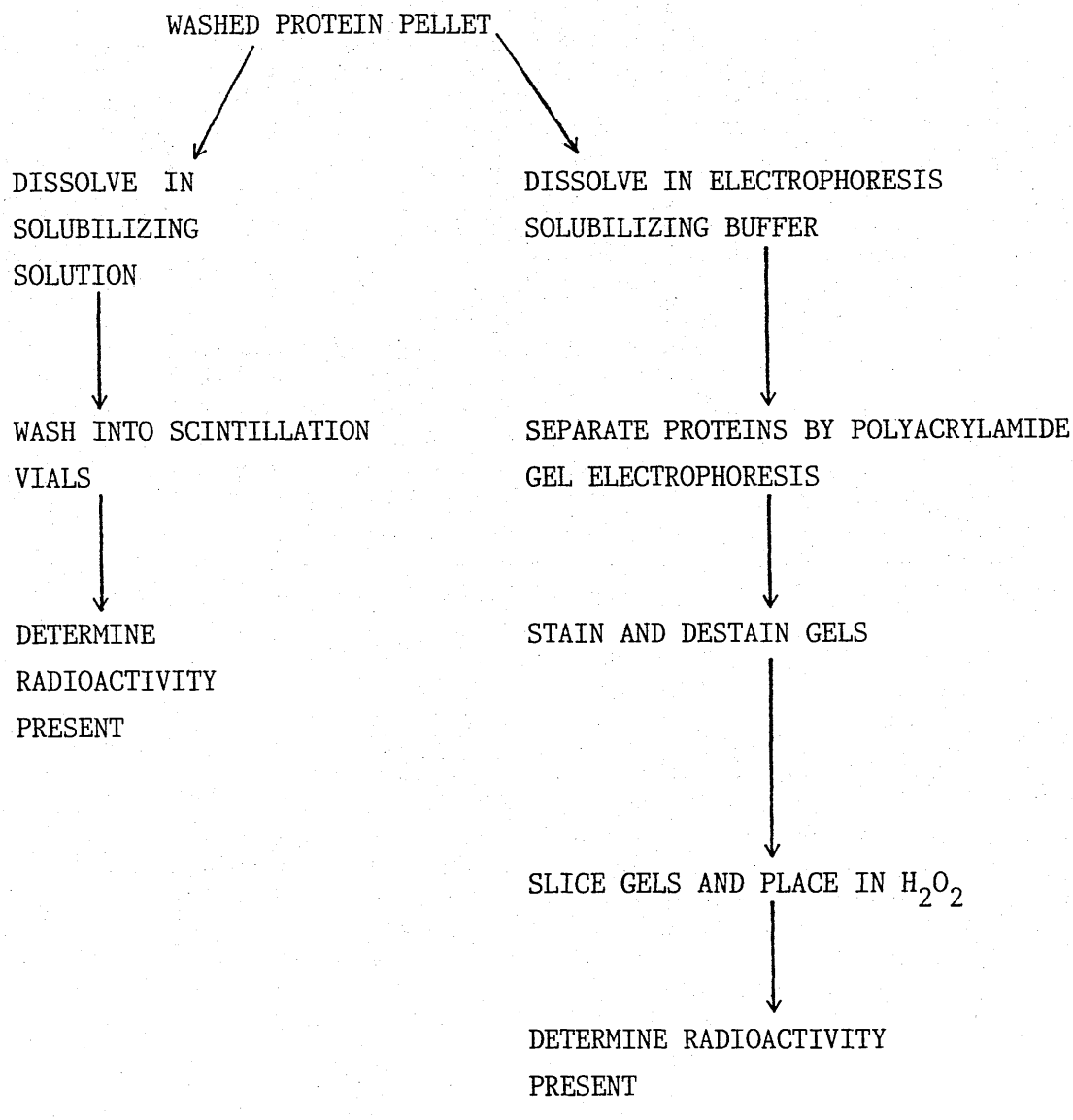
At the end of each incubation, ganglia were transferred to 5 ml test tubes containing 100 µl of ice-cold 6 mM-Tris/maleate, pH 7.4, 2 mM-EGTA, and immediately homogenized with a close-fitting teflon pestle. Homogenates (70 µl) were quickly transferred to Eppendorf vials containing 70 µl of 10% trichloroacetic acid (TCA). Aliquots were taken for protein determination (2 x 10 µl). The TCA precipitated proteins were collected by centrifuging at 10,000 g for 10 minutes. The resulting pellets were washed twice with 5% TCA, and incubated at 37 °C with chloroform/methanol, 2:1 (containing 0.3% concentrated HCl v/v) twice for 10 minutes. On both occasions the vials were centrifuged at 10,000 g for 5 minutes before removing the chloroform/methanol. The resulting pellets were resuspended in 200 µl, 0.2 M-$Na_2 HPO_4$, pH 6.4 containing 20 µg of Ribonuclease A, and incubated for 2 hours at 37 °C. At the

* vials were kept open

Figure 6: Summary of Protein Pellet Extraction



SEE OVER


WASHED PROTEIN PELLET
DISSOLVE IN SOLUBILIZING SOLUTION
WASH INTO SCINTILLATION VIALS
DETERMINE RADIOACTIVITY PRESENT
DISSOLVE IN ELECTROPHORESIS SOLUBILIZING BUFFER
SEPARATE PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS
STAIN AND DESTAIN GELS
SLICE GELS AND PLACE IN H_2O_2
DETERMINE RADIOACTIVITY PRESENT

end of the incubation 10% TCA (200 µl) was added and precipitates were again collected at 10,000 g for 10 minutes. Pellets were washed twice with 5% TCA and once with di-ethyl ether, yielding washed protein pellets. These were dissolved by adding 0.1 M-NaOH, 2% SDS w/v (200 µl) to the Eppendorf vials and allowing them to dissolve overnight at 30 ^{o}C. The solubilized pellets were transferred together with 3 x 100 µl washes with NaOH/SDS (as above), to scintillation vials. Scintillant (4 ml) was added to the vials and they were taken for liquid scintillation counting. This was carried out for 4 minutes per vial on the open channel setting. Scintillant was composed of toluene/Triton X-100 2:1 v/v containing 0.8% PPO w/v. Protein-bound phosphate was expressed as counts per minute (cpm) present in the 'washed' pellet per mg of protein.

2:3:3 Rod-gel Electrophoresis of Intact Ganglia proteins (Figure 6)

2:3:3:1 Sample Preparation

In these experiments the washed pellet obtained at the end of the protein extraction, was dissolved in 50 µl electrophoresis buffer, consisting of; 0.062 M-Tris/HCl, pH 6.8, 1% SDS w/v, 10% glycerol v/v, 2.5% 2-mercapto-ethanol and 0.005% bromophenol blue. The mixture was boiled for 2 minutes prior to electrophoresis and aliquots (20-40 µl) containing 30 µg of protein were loaded onto the gels.

2:3:3:2 Preparation of Rod-gels

Gels were prepared from the following stock solutions, which were stored at 4 ^{o}C; 30% Acrylamide/0.8% methylene-bis acrylamide w/v, 1.12 M Tris-HCl, pH 8.8, and 10% SDS w/v. Normally, 30 ml of gel solution was prepared, to give an acrylamide concentration of 12.5% which was found to give the best overall separation. Acrylamide stock, (12.5 ml) Tris-HCl stock (10 ml) and distilled water (7.2 ml) were mixed in a buchner flask, and de-gassed by attaching the flask to a vacuum pump. SDS stock (0.3 ml), TEMED, (15 µl) and 40 mg of solid freshly weighed ammonium persulphate was then added and the solution thoroughly mixed. This solution was poured into glass tubes (4 mm internal diameter, and 110 mm long) to a height of 90 mm. The bottom of the tubes had been sealed with parafilm. The surface of the gel solution was carefully overlaid with 0.1% SDS w/v, and allowed to set for at least one hour.

2:3:3:3 Electrophoresis

The unpolymerized layer at the top of the gel, and the parafilm from the bottom the tubes were removed and the tubes placed into the rod-gel apparatus, similar to that of Maurer, (1971). The apparatus was filled with electrophoresis buffer (Tris, 0.025 M; Glycine, 0.192 M; SDS 0.1% w/v; pH 8.3), and the gels pre-run at a constant current of 1 mA/tube for 15 minutes. Samples (prepared as in section 2:3:3:1) were loaded onto the top of the gels, below the surface of the buffer, with a Hamilton syringe. The apparatus was switched on again and the current maintained at 1 mA/tube until the sample completely

entered the gel. The current was then increased to 3 mA/tube, and electrophoresis (from -ve to +ve electrodes) was continued until the tracking dye was approximately 5 mm from the bottom of the gel. The gel tubes were taken out of the electrophoresis apparatus and the gels were removed by injecting water down the sides of the tube, placing a pasteur pipette teat at one end and slowly squeezing the gel out. Gels were then pierced with a stainless-steel pin to mark the tracking dye front and placed into test tubes containing staining solution (Coomassie blue, 0.1% w/v; methanol, 50%; water, 40%; acetic acid, 10%) for one hour at 37 ^{o}C. Gels were destained in water, 60%; methanol, 30%; acetic acid, 10% v/v for at least 24 hours. The destaining solution was changed twice within the first hour.

2:3:3:4 Slicing of Gels

Stained gels were frozen solid, by covering them with finely crushed solid CO_2 and sliced into 2 mm slices. The slices were placed into scintillation vials containing 0.5 ml hydrogen peroxide (20 volumes), and allowed to dissolve overnight at 37 ^{o}C. Scintillant (4 ml) was added to each vial, and the contents thoroughly mixed, before determining radioactivity by liquid scintillation counting, (section 2:3:2).

2:3:4 Slab-gel Electrophoresis of proteins from Intact Ganglia

2:3:4:1 Incubation of ganglia and preparation of samples for electrophoresis

Ganglia were incubated for 22 hours as described in section 2:3:1 except with 170 µCi/ml of ^{32}Pi. In other experiments all the ganglia to be used in each experiment were pre-incubated together in a bijous vial, containing iso-osmolar saline (Section 2:2), with 170 µCi/ml ^{32}Pi for 3 hours at 30 °C. At the end of the pre-incubation period, ganglia were washed in fresh iso-osmolar saline maintained at 30 °C. They were then transferred to Eppendorf vials (4/vial), containing iso-osmolar saline (0.5 ml) with the appropriate final concentration of octopamine or dibutyryl cyclic AMP, and re-incubated for 10 minutes at 30 °C.

Incubations were terminated by transferring ganglia to 5 ml test-tubes containing ice-cold 6 mM-Tris/maleate, 2 mM-EGTA, pH 7.4 (400 µl), and homogenized (section 2:3:2). Homogenates (300 µl) were quickly transferred to Eppendorf vials containing 70 µl of solubilizing buffer (consisting of; 286 mM-Tris, 266 mM-glycine, 5.7% SDS w/v, 28.5% sucrose w/v, 5.7% freshly added 2-mercaptoethanol v/v, and 0.03% bromophenol blue), and placed in a boiling water bath for 2 minutes. Samples were stored at -20 °C and electrophoresis was carried out within 20 hours of preparation. Aliquots (3 x 20 µl) of the remaining homogenate were taken for protein determination.

2:3:4:2 Preparation of Slab-gels and Electrophoresis

Gel solution with a final acrylamide concentration of 10%, was prepared from stock solutions described in section 2:3:3:2 in

the following proportions;

10 ml-acrylamide, 10 ml-Tris/HCl, pH 8.8, 9.6 ml-distilled water, and degassed as before prior to adding 0.3 ml-SDS, 15 µl-TEMED and 150 µl -ammonium persulphate (10% w/v). This gel solution was poured into the slab-gel apparatus (as described by Takacs, 1979), which consisted of 2 glass plates, (20 cm x 20 cm) separated by 3 strips of perspex (8 mm wide and 1.5 mm thick) one along the bottom edge and the other 2 at the sides. The plates were clamped together using ordinary foldback office clips and sealed by running molten agar (1% w/v agarose) around the outside edges. The gel solution was poured between the plates to a level of approximately 2 cm from the top, gently overlaid with iso-butanol, and allowed to set for one hour. After one hour the butanol and unpolymerized layer were removed and the surface of the gel was rinsed with electrophoresis buffer (section 2:3:3:3). Before pouring stacking-gels, a perspex comb to form wells (80 µl volume) in the stacking gel was inserted between the plates, leaving a space of 1.0 cm between the fingers of the comb and the surface of the separating-gel. Stacking gels were prepared from stock solutions (section 2:3:3:2, except buffer, which was; 0.5 M-Tris/HCl, pH 6.8) in the following proportions; 1.5 ml-acrylamide, 3.75 ml-Tris/HCl, 9.45 ml-distilled water, 0.15 ml-SDS, 10 µl-TEMED, and 150 µl-ammonium persulphate (10% w/v). The stacking-gels were allowed to set for half an hour after which the comb, bottom clips and bottom perspex spacer were removed and the plates clamped to the slab-gel electrophoresis apparatus which was sealed with grease and filled with electrophoresis buffer, (section 2:3:3:3).

Samples were placed into the wells below the surface of the buffer with a Hamilton syringe. Between 30-50 µl of sample per well was used, adjusted to contain 35 µg of protein. Electrophoresis was then carried out at a constant current of 40 mA until the tracking dye was approximately 1 cm from the bottom of the gel. Electrophoresis was carried out from -ve(origin) to +ve(bottom).

At the end of electrophoresis, the plates were removed and the outer surface rinsed with water to remove any radioactive contamination from the lower well buffer. Plates were levered apart using a sturdy spatula and stacking gels removed by cutting them away from the separating gels with a scalpel. The separating gels were lifted gently from the plates and placed in 5% TCA (w/v) for 30 minutes at 95 ^{o}C, after which they were transferred to 5% TCA at 30 ^{o}C for one hour, then 10% acetic acid (v/v) at 30 ^{o}C for one hour. They were then stained as described for rod-gels, (section 2:3:3:3) and destained in a solution of water 55%, methanol 35% and acetic acid 10% (v/v). The final soaking for one hour in destaining solution contained 1% glycerol to prevent gels from reticulating after drying.

2:3:4:3 Autoradiography of Slab-gels

Gels were placed on Whatman 3 mm paper, overlaid with cling film and dried under vacuum for 3 hours on a Bio-Rad gel drier, of which the heating unit was switched on for the first hour only. Autoradiography was carried out by placing a sheet of Kodak X-OMAT AR film and an Ilford fast tungstate intensifying screen on top of the dry gels and clamping them together in a

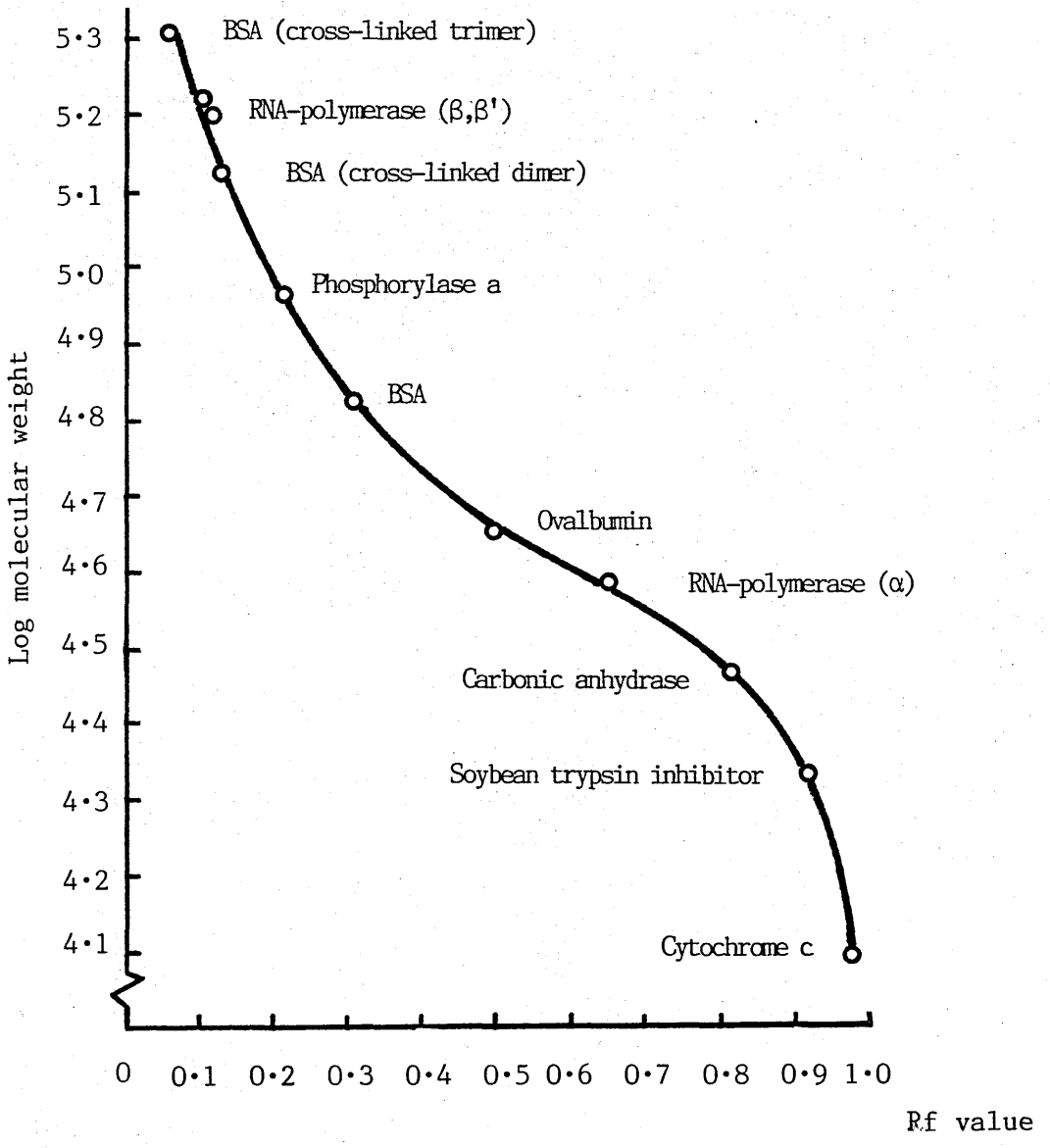
plastic cassette which was wrapped in a sheet of aluminium foil. The covered cassette was sealed in a plastic bag and placed in a deep freeze at ≤ -70 °C for 2-3 days. Before developing the films, cassettes were allowed to thaw out for at least 2 hours at room temperature.

Quantitative measurement of the radioactivity present in the gels was achieved by cutting out the individual bands and placing them in scintillation vials with hydrogen peroxide overnight at 37 °C and then taken for liquid scintillation counting as in section 2:3:3:4.

2:3:4:4 Molecular Weight determination of Proteins

Protein standards with well-characterized molecular weights were routinely run on all gels. The molecular weights of unknown proteins were estimated by comparing their mobilities to those of the standards. This was achieved by plotting the Rf value of each molecular weight standard against the log of its molecular weight for each gel (Figure 7). The molecular weights of unknown proteins were then extrapolated from the graph.

Figure: 7 Calibration Curve for the Determination of Protein Molecular Weights in Polyacrylamide Gels



Proteins were subjected to electrophoresis on 10% polyacrylamide/SDS slab-gels (section 2:3:4:2).

2:4 Incorporation of ^{32}P into Nucleotides in Intact Ganglia

2:4:1 Preparation of acid soluble fraction

Ganglia were prepared and incubated in iso-osmolar saline as described in section 2:3:1. At the end of each incubation, ganglia were rapidly removed from the incubation vials and washed quickly in 3 changes of ice-cold iso-osmolar saline before homogenizing (see section 2:3:2) in 90 µl 10% TCA. Homogenates were centrifuged at 10,000 g for 10 minutes and the supernatant transferred to an Eppendorf vial, and 10 µl of a solution containing ATP, ADP, AMP, GDP, GTP, (120 µg of each) were added as standards.

2:4:2 Chromatography of Acid Soluble Fraction

Aliquots (5 µl) of the acid soluble tissue fraction containing standards (as in section 2:4:1), were chromatographed on PEI cellulose, plastic TLC sheets (20 cm long). The sheets were placed in beakers sealed with aluminium foil and chromatography in 0.85 M-KH_2PO_4, pH 3.4, allowed to continue until the solvent front reached the top of the sheets. Chromatograms were then removed from the beakers and thoroughly dried before placing Kodak KD 54-T x-ray film onto them, attached with tape and then wrapped in aluminium foil. Chromatograms were autoradiographed for 7-10 days, after which the x-ray film was developed to locate the distribution of radioactivity. Chromatograms were visualized under short-wave ultra-violet light to locate standards. The radioactive areas on chromatograms were cut out and placed in scintillation vials with 4 ml of scintillant (as section 2:3) for counting. The radioactivity incorporated

into nucleotides was determined by measuring the radioactivity in areas which co-chromatographed with standards.

The recovery of radioactivity from the PEI cellulose sheets was assessed, and cutting out the radioactive areas yielded 91.1% $\pm$ 2.3 recovery, and scraping-off the cellulose layer resulted in 86.1% $\pm$ 2.1 recovery.

2:5 Protein Phosphorylation in Homogenized Tissue

2:5:1 Preparation of Tissue Fractions

2:5:1:1 Soluble (Sf) and Particulate (Pf) Fractions (summarized in Figure 8)

Cerebral ganglia were obtained as described in section 2:2 and homogenized in ice-cold 6 mM-Tris/maleate, 2 mM-EGTA, pH 7.4, (60 mg/ml in the measurement of total protein phosphorylation and 120 mg/ml in the measurement of specific protein phosphorylation). This homogenate was transferred to Eppendorf vials and centrifuged at 10,000 g for 10 minutes at 4 ^{o}C. The supernatant (S_A) was transferred to another Eppendorf vial and the pellet (P_A) was resuspended in the original volume of homogenizing buffer, after which both S_A and P_A were re-centrifuged at 10,000 g for 10 minutes at 4 ^{o}C. The supernatant (S_B) obtained from re-centrifuged S_A was transferred to another vial and this was referred to as the soluble fraction (Sf), the pellet was discarded. The supernatant from re-centrifuged P_A was also discarded and the pellet P_B was resuspended in half the original volume of homogenizing buffer, this was referred to as the particulate fraction (Pf).

2:5:1:2 Subcellular Fractionation (summarized in Figure 9)

Cerebral ganglia were removed (section 2:2), placed on a nylon disc and chopped with one pass of a Mickle tissue slicer set at 0.2 mm. The chopped tissue was transferred to glass tubes and homogenized in ice-cold 0.25 M-sucrose/100 mM-Tris/HCl, pH 7.4, (100 mg/ml) with 10 passes of a hand-held close-fitting teflon pestle. Homogenates were transferred to Eppendorf vials and centrifuged at 700 g for 10 minutes at 4 ^{o}C. The supernatant

Figure: 8 Preparation of Soluble (Sf) and Particulate (Pf) Fractions

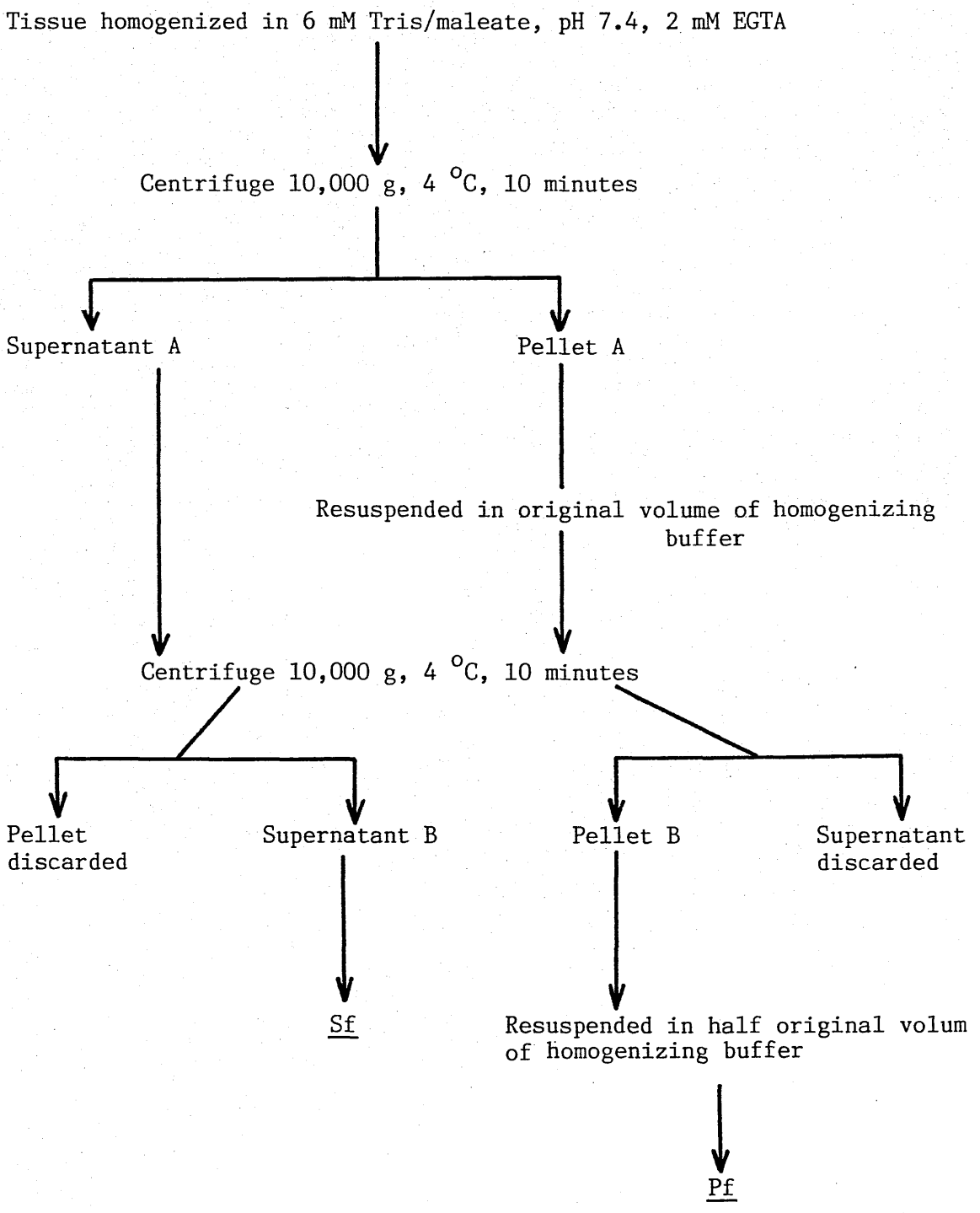
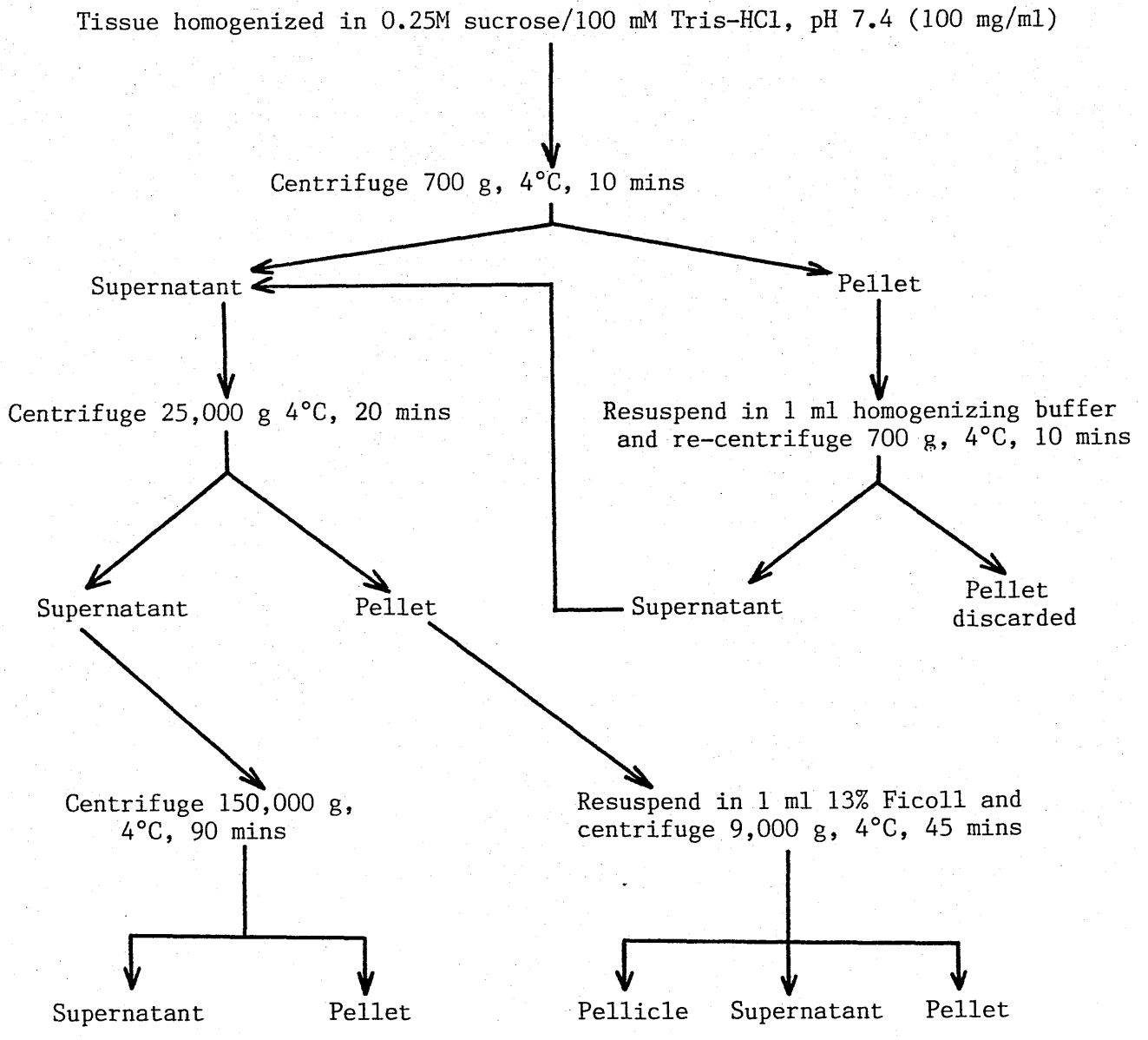


Figure: 9 Subcellular Fractionation



was collected and the pellet resuspended in homogenizing buffer (1 ml/100 mg original wet weight of tissue) and re-centrifuged at 700 g as previously. The pellet obtained from re-centrifugation was discarded and the supernatant pooled with that of the previous 700 g centrifugation. Pooled supernatants were centrifuged at 25,000 g for 20 minutes at 4 ^{o}C to obtain a 25,000 g pellet and supernatant. The pellet was either resuspended in 400 µl of 6 mM-Tris/maleate, 2 mM-EGTA, pH 7.4, or further fractionated. The 25,000 g pellet was transferred to a pre-chilled glass tube and the vial washed with 200 µl of homogenizing buffer. Thirteen per cent Ficoll/100 mM-Tris/HCl, pH 7.4 (1 ml) was added to the glass tubes and pellets resuspended with 5 passes of a ground glass pestle. The resuspended pellet was equally distributed between 4 microvials. These were held in position in a swing-out rotor by nylon inserts with a hole bored out of the centre the same diameter as the microvials. These were centrifuged at 9,000 g for 45 minutes at 4 ^{o}C, after which a pellicle and pellet were obtained. The pellicles floating at the top of the Ficoll were removed with a Pasteur pipette (approximately the top 20 µl-30 µl) and placed in an Eppendorf vial to which was then added 1.5 ml of homogenizing buffer. This was centrifuged at 10,000 g for 5 minutes at 4 ^{o}C, after which the supernatant was discarded and the pellet was resuspended in 6 mM-Tris/maleate, 2 mM-EGTA, pH 7.4 (50 µl) and referred to as the synaptosomal fraction. The remaining Ficoll in the microvials was removed and each pellet rinsed twice with 100 µl homogenizing buffer before being resuspended in 20 µl of 6 mM-Tris/maleate as above and pooled in an Eppendorf vial. This was

referred to as the mitochondrial fraction.

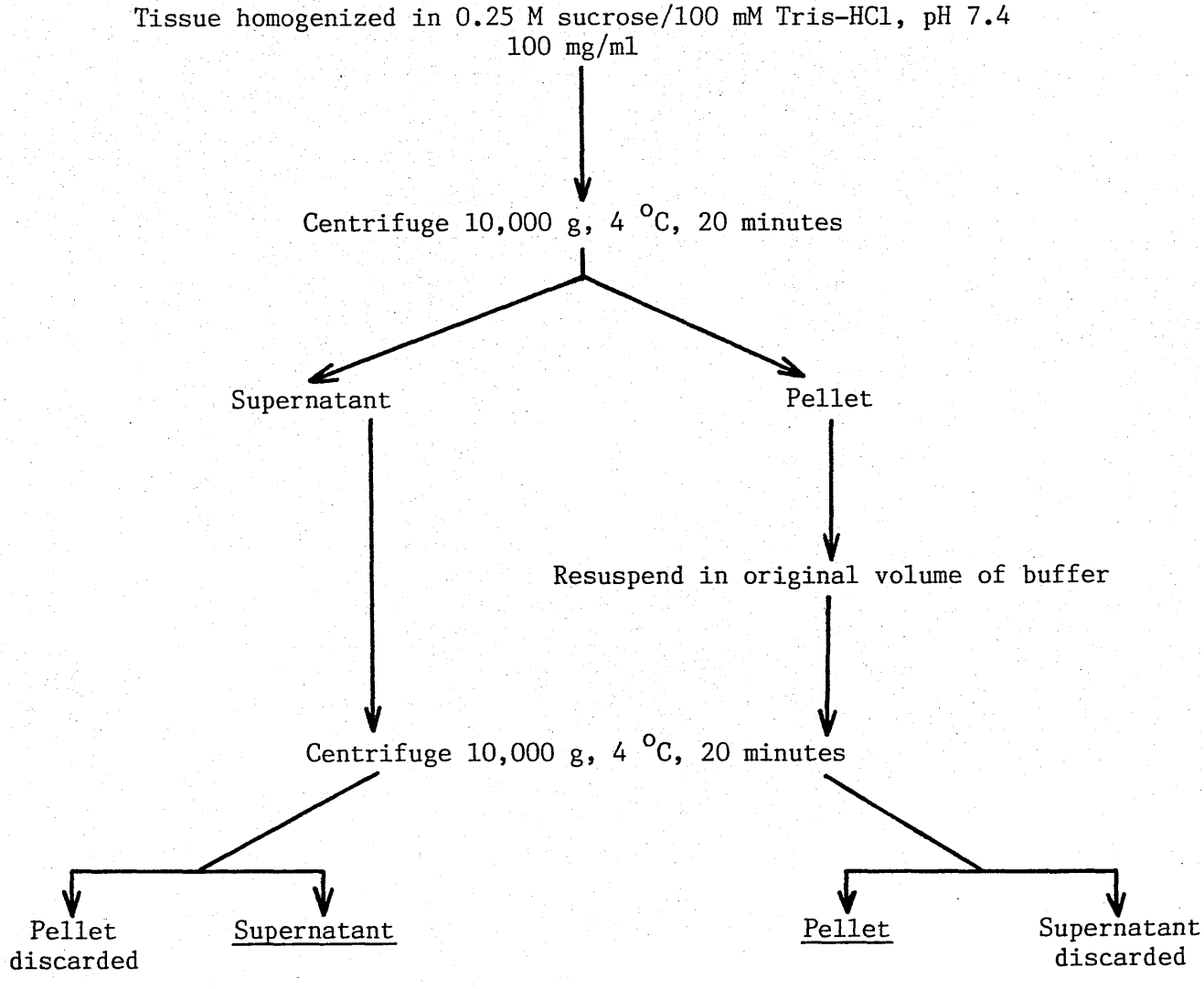
The 25,000 g supernatant was placed in 10 ml polycarbonate tubes and centrifuged at 150,000 g for 90 minutes at 4 ^{o}C. The supernatant obtained was used directly in the measurement of protein phosphorylation. The pellet was rinsed twice with 1 ml homogenizing buffer and resuspended in 100 µl 6 mM-Tris/maleate, 2 mM-EGTA, pH 7.4.

2:5:1:3 Crude Synaptosomes

Crude synaptosomes were prepared by a method similar to that of Kelly, 1981). Cerebral ganglia were removed, chopped and homogenized (100 mg/ml) as in section 2:5:1:2. Homogenates were centrifuged at 3,000 g for 10 minutes at 4 ^{o}C. The supernatant was collected, and the pellet resuspended in 1 ml of homogenizing buffer and re-centrifuged. The supernatants were combined and re-centrifuged at 30,000 g for 30 minutes at 4 ^{o}C. The supernatant was collected and used directly. The 30,000 g pellet was resuspended in 1 ml homogenizing buffer and re-centrifuged at 30,000 g. The supernatant was discarded and the pellet was resuspended in 6 mM-Tris/maleate, 2 mM-EGTA, pH 7.4, (250 µl/100 mg of ganglia) and freeze thawed 5 times in methanol/solid CO_2.

2:5:1:4 10,000 g Fractions prepared in 0.25 M sucrose (summarized in Figure 10 These were obtained by homogenizing ganglia (100 mg/ml) as in section 2:5:1:2 in 0.25 M sucrose/100 mM-Tris/HCl, pH 7.4. Homogenates were centrifuged twice at 700 g for 10 minutes at

Figure: 10 10,000 g Fractions Prepared in 0.25 M Sucrose



4 °C, (pellets were resuspended in 1 ml of buffer). Pellets obtained after the second 700 g centrifugation were discarded and pooled supernatants centrifuged at 10,000 g for 10 minutes at 4 °C. The supernatants were collected for assay and the pellets were resuspended in homogenizing buffer (1 ml) and re-centrifuged at 10,000 g after which they were resuspended in 6 mM-Tris/maleate, 2 mM-EGTA, pH 7.4, (250 µl/100 mg of ganglia).

2:5:2 Measurement of Total Protein Phosphorylation

Reaction mixtures (150 µl) were pre-incubated for 5 minutes at 30 °C in Eppendorf vials and reactions started by adding 50 µl of the appropriate tissue fraction, (crude homogenate contained 60 mg tissue/ml, see Section 2:5:1:1). The normal reaction mixture contained the following final concentrations; 80 mM-Tris/maleate, pH 7.4, 10 mM-theophylline, 2 mM-$MgSO_4$, 0.7 mM-EGTA, 10 µM-ATP (1-3 µCi [γ^{32}P] ATP/incubation), and the relevant concentration of test substance. Reaction mixtures were incubated for 1 minute (unless otherwise stated) and terminated by adding 10% TCA (200 µl). Precipitates were collected by centrifuging at 10,000 g for 10 minutes and pellets were extracted to yield 'washed' protein pellets as described in section 2:3:2.

2:5:3 Measurement of Specific protein phosphorylation

This was achieved by incubating tissue fractions with [γ^{32}P] ATP followed by Polyacrylamide/SDS gel electrophoresis.

Phosphorylation reactions were carried out in Eppendorf vials. Reaction mixture (145 µl) was pre-incubated for 5 minutes at 30 °C and the reaction started by adding 20 µl of the relevant tissue fraction (crude homogenate contained 120 mg tissue/ml, see Section 2:5:1:1). The normal reaction mixture contained the following final concentrations; 80 mM-Tris/maleate, pH 7.4, 10 mM-theophylline, 2 mM-$MgSO_4$, 0.7 mM-EGTA, 10 µM-ATP (6-8 µCi [γ^{32}P] ATP/incubation) and the appropriate concentration of test substance. Reactions were terminated after 1 minute

(unless otherwise stated) by adding 35 µl of electrophoresis solubilizing buffer, (Section 2:3:4:1) and vortexing, before boiling for 2 minutes. Samples were then subjected to electrophoresis on polyacrylamide slab gels and autoradiographed as described in sections 2:3:4:2 and section 2:3:4:3 except that the treatment with hot TCA was ommited and gels were directly stained after electrophoresis.

2:5:4 Crude Homogenate incubated with [γ^{32}P] ATP before separating into Sf and Pf

2:5:4:1 Total Protein Phosphorylation

Crude homogenates, prepared in 6 mM-Tris/maleate, pH 7.4, 2 mM-EGTA, (Section 2:5:1:1), were incubated as described in section 2:5:2 for one minute and immediately centrifuged at 10,000 g for 10 minutes at 4 ^{o}C. The supernatant was removed and re-centrifuged at 10,000 g as before. The pellet was resuspended in ice-cold 6 mM-Tris/maleate, pH 7.4, 2 mM-EGTA (200 µl) and also re-centrifuged. The re-centrifuged supernatant was removed and 100 µl quickly placed into 100 µl of 10% TCA. The supernatant from the re-centrifuged pellet was removed and the pellet was resuspended in 10% TCA (200 µl). TCA precipitates were collected by centrifuging at 10,000 g and the pellets extracted to obtain 'washed' protein pellets as described in section 2:3:2.

2:5:4:2 Specific Protein Phosphorylation

Sf and Pf were prepared after incubating crude homogenate with [γ^{32}P] ATP as described in section 2:5:4:1. Re-centrifuged

supernatant (100 µl) was quickly transferred to Eppendorf vials containing 21 µl electrophoresis solubilizing buffer, (Section 2:3:4:1). The re-centrifuged pellet was resuspended in 30 µl of electrophoresis solubilizing buffer diluted 1 + 4.7 with 80 mM-Tris/maleate, pH 7.4. All samples were boiled for 2 minutes immediately after adding solubilizing buffer. Samples were then taken for polyacrylamide/SDS slab-gel electrophoresis, and autoradiographed, (Sections 2:3:4:2 and 2:3:4:3).

2:5:5 Pronase Treatment of Proteins

Homogenates prepared in 6 mM-Tris/maleate, pH 7.4, 2 mM-EGTA (section 2:5:1:1) were incubated in Eppendorf vials as described in section 2:5:2. Incubations were terminated by adding an equal volume of 10% TCA and precipitates collected by centrifuging at 10,000 g for 10 minutes. The pellets were extracted to obtain 'washed' protein pellets (section 2:3:2). The pellets were resuspended in 0.2 M-NaH_2PO_4, pH 6.4, (200 µl) with or without 20 µg of pronase and incubated for 24 hours at 37 °C. At the end of the incubation either 10% TCA (200 µl) or 45 µl of electrophoresis solubilizing buffer (section 2:3:4:1) was added to the vials. Vials to which TCA had been added were centrifuged at 10,000 g for 10 minutes and the radioactivity in both pellet and supernatant determined as described in section 2:3:2. The vials to which solubilizing buffer had been added were immediately placed in a boiling water bath for 2 minutes. Samples were then taken for gel electrophoresis (section 2:3:4).

2:6 Determination of ATPase Activity

ATPase activity was determined by incubating tissue fractions with ATP and measuring the formation of inorganic phosphate.

2:6:1 Incubation and Preparation of samples

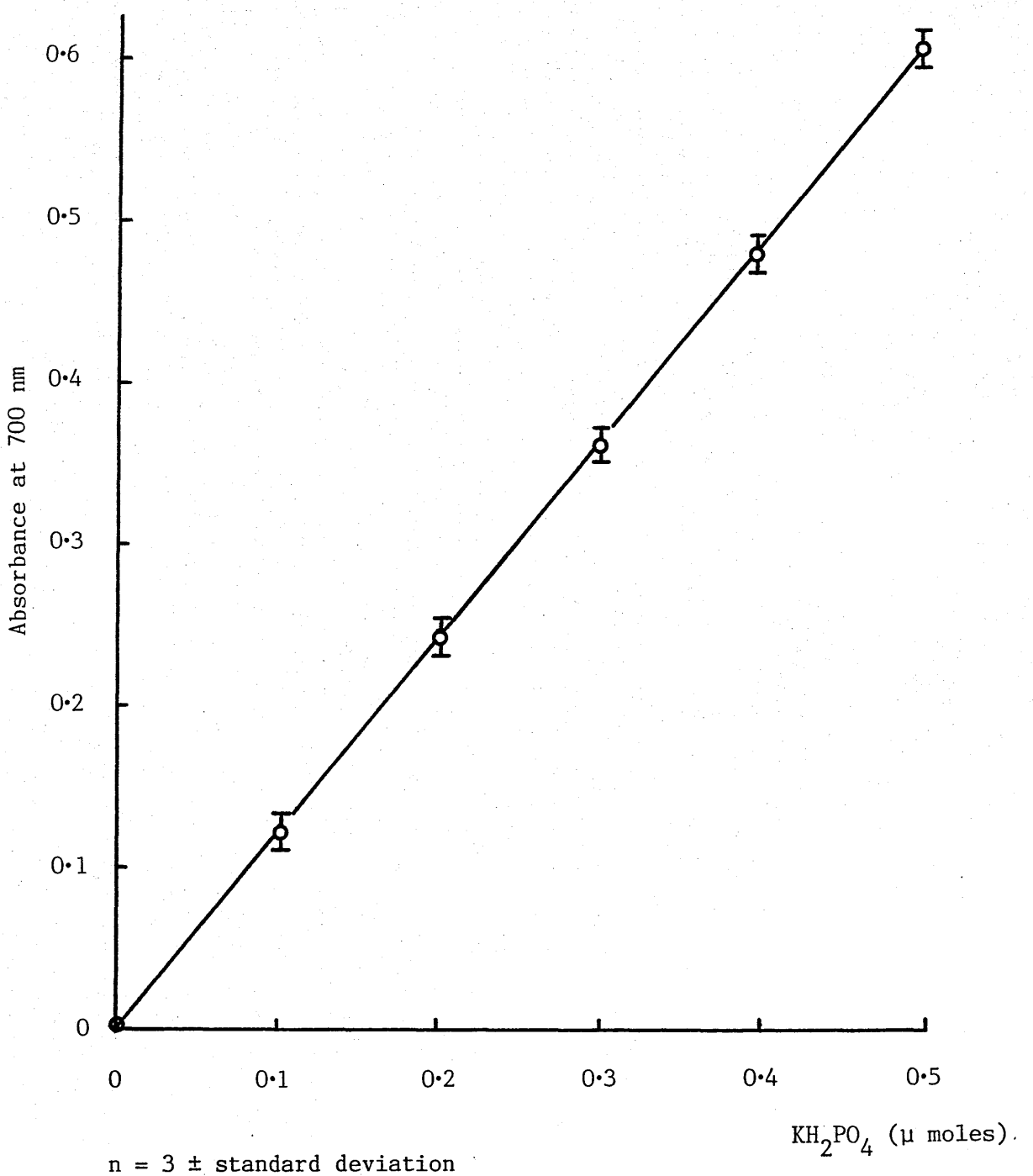
Incubations were carried out in Eppendorf vials. Reaction mixture (225 µl) containing 125 µl of the relevant tissue fraction prepared as described in section 2:5:1:1 was pre-incubated for 2 minutes at 30 °C. Reactions were started by adding ATP (25 µl) and contained the following final

concentrations; 80 mM-Tris/maleate, pH 7.4, 10 mM-theophylline, 2 mM-$MgSO_4$, 0.7 mM-EGTA and 2 mM-ATP. Incubations were terminated by adding 10% TCA (250 µl) for zero-time incubations, TCA was ~~added~~ before ATP. The precipitated protein was removed by centrifugation (10,000 g for 10 minutes) and 2 x 200 µl aliquots of the supernatant were transferred from each vial to 10 ml test-tubes for the assay of inorganic phosphate.

2:6:2 Phosphate assay

Molybdate reagent (1.5 ml) which was prepared fresh immediately before each assay in the following proportions; 2:1:1 of 5% sodium molybdate, 10 M sulphuric acid, distilled water, was added to the test-tubes followed by 0.5 ml 1% Metol/3% sodium metabisulphate. Tubes were allowed to stand at room temperature for 30 minutes after which the absorbance was measured at 700 nm within one hour of adding the reagents. Absorbances for samples were compared to absorbances for standard concentrations of KH_2PO_4, (Figure 11 shows a typical calibration curve for KH_2PO_4).

Figure: 11 Calibration Curve for the Determination of Inorganic Phosphate



n = 3 ± standard deviation

2:7 Protein Determination

2:7:1 Preparation of samples

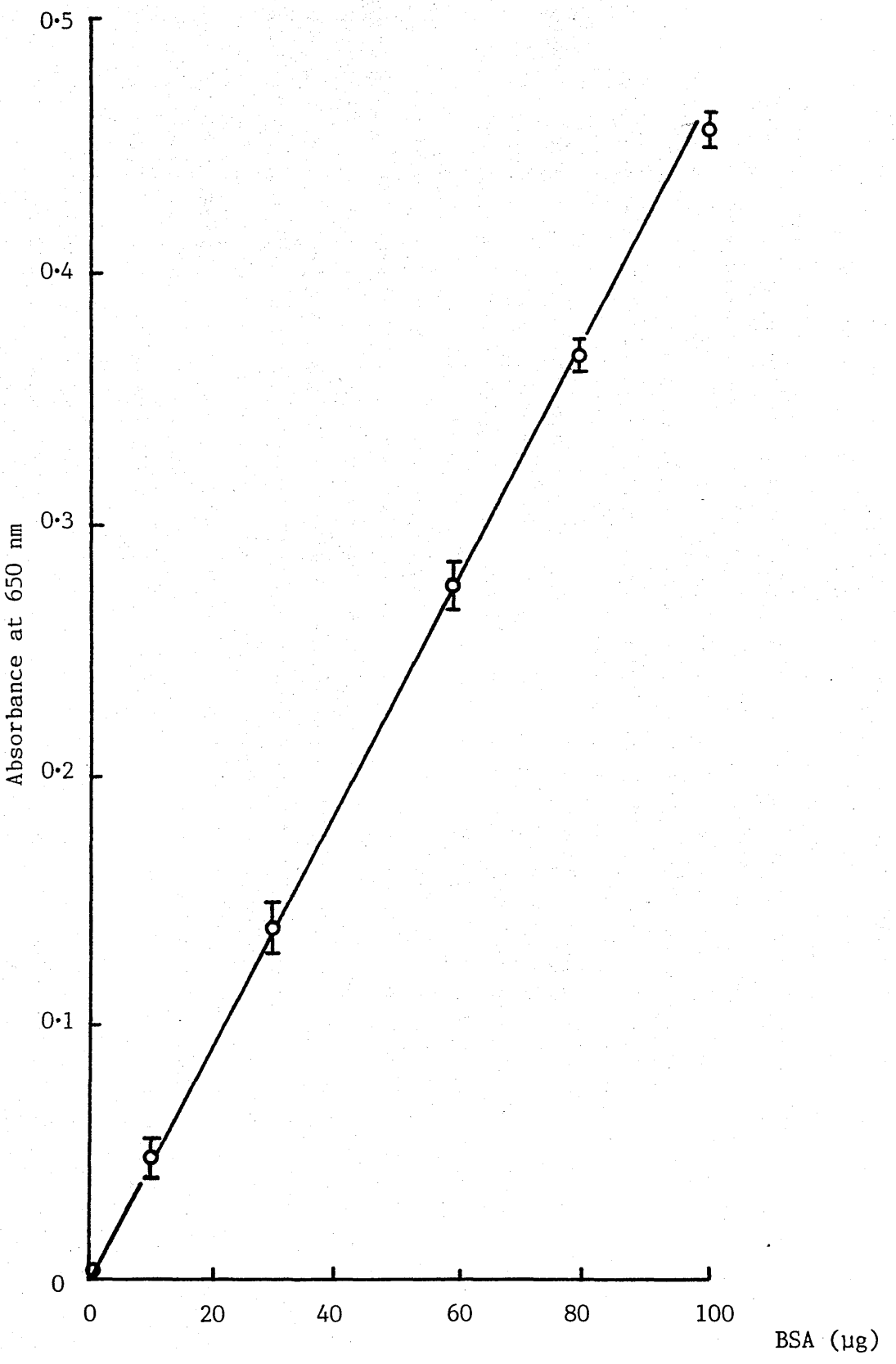
In experiments where total protein phosphorylation was studied aliquots of homogenized tissue were extracted in the same manner as the experimental homogenates, (section 2:3:2). 'Washed' protein pellets were solubilized by adding 0.1 M-NaOH/2% SDS to the Eppendorf vials overnight at 37 ^{o}C. Vials were vortexed and spun briefly (10 seconds) to remove droplets from the walls of the vial before removing 100 µl of liquid for protein assay.

In other experiments 10 µl of tissue homogenate were taken for protein assay.

2:7:2 Protein Assay

Protein samples (100 µl) or made up to 100 µl with distilled water, were placed in 10 ml test-tubes and 0.1 M-NaOH/2% SDS (1 ml) was added. Tubes were placed in a water bath for 30 minutes at 37 ^{o}C before adding 2 ml of copper sulphate reagent, which was prepared immediately before use from the following stock solutions; 0.5% $CuSO_4$ (w/v)/1% trisodium citrate (1 part) and 0.1 M-NaOH/2% Na_2CO_3 (w/v)/0.2% SDS (50 parts). Tubes were then allowed to stand at room temperature for 10 minutes before adding water/Folin-Ciocalteau reagent 2:1 (0.5 ml) and left for at least a further 30 minutes before measuring the absorbance at 650 nm. Absorbances obtained for unknown samples were compared to known amounts of BSA in the range 0-100 µg, (Figure 12 shows a typical calibration curve for BSA).

Figure: 12 Calibration Curve for Protein Determination



n = 3 ± standard deviation

2:8 Statistical Analysis

Values were calculated for unequal variance, and probability values for the appropriate degrees of freedom were obtained from statistical tables, (Fisher & Yates, 1953). The null hypothesis was rejected at the 5% level of probability, i.e. $U_A \neq U_B$ when $P \leq 0.05$ and the difference deemed to be significant.

3 RESULTS

3:1 Phosphorylation in Intact Ganglia

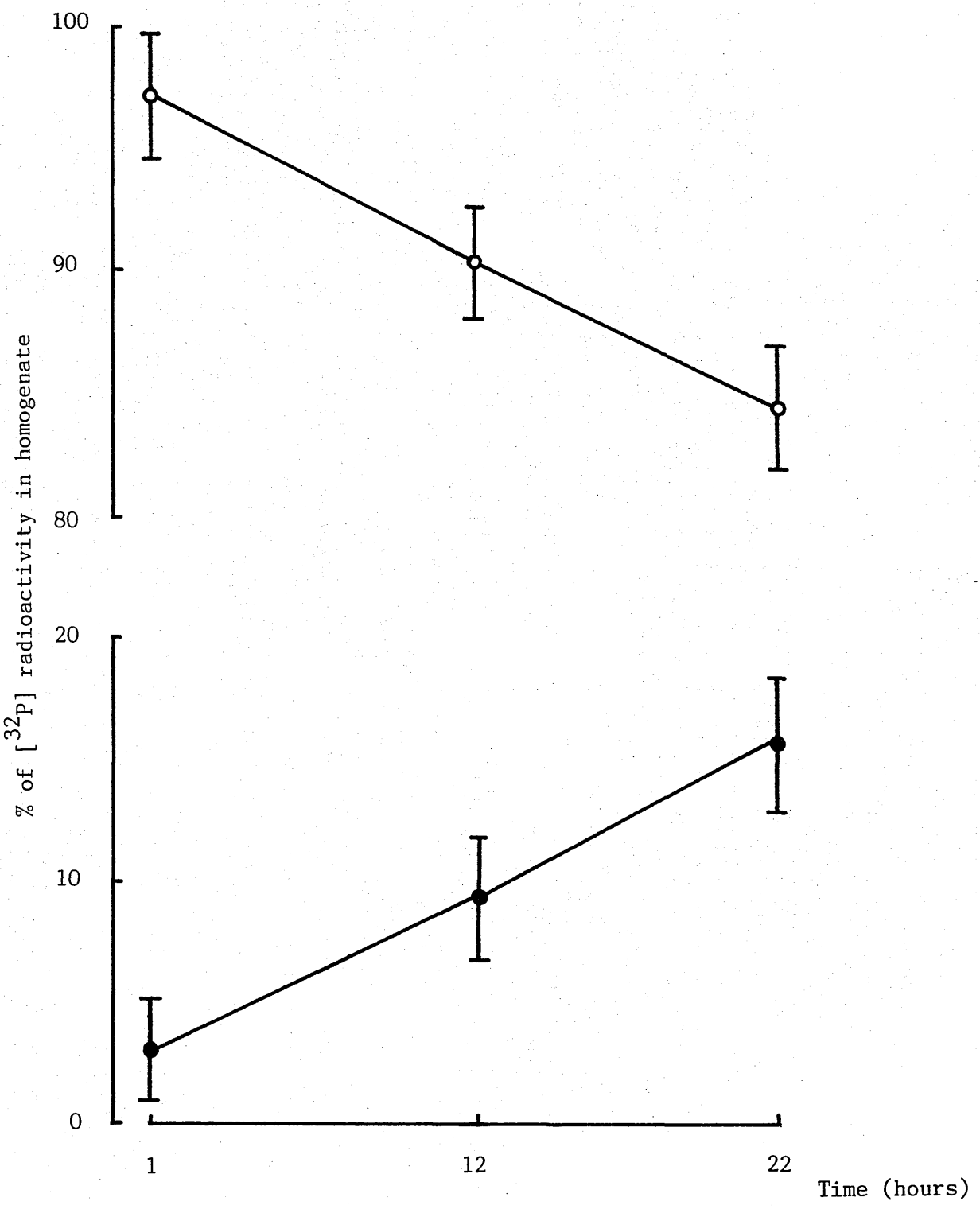
3:1:1 Preliminary Studies

Incubation of intact ganglia with ^{32}Pi followed by homogenizing with TCA resulted in the major proportion of radioactivity remaining in the TCA soluble fraction, (Figure 13). Radioactivity in the TCA insoluble material increased from 2.6% $\pm$ 1.8 for ganglia incubated for 3 hours, to 15.4% $\pm$ 2.7 after 22 hours.

Figures 14 and 15 show the distribution of radioactivity on extracting the TCA insoluble material from ganglia incubated with ^{32}Pi for 3 and 22 hours respectively, (the lower half of both figures are on the same scale for direct comparison). Washing the initial TCA insoluble material with 5% TCA removed relatively smaller amounts of radioactivity. Treatment of the pellet with chloroform/methanol removed additional radioactivity in the first wash, and less in the second, indicating the removal of phospholipids. Treatment of the resuspended pellet with ribonuclease released a further amount of radioactivity, to a greater extent in pellets from ganglia incubated for 22 hours than those incubated for 3 hours. This indicates that much of the radioactivity remaining after the chloroform/methanol treatment in pellets from ganglia incubated for 22 hours, was incorporated into RNA. In contrast, very little radioactivity was incorporated into RNA after 3 hours. The radioactivity remaining after ribonuclease and TCA treatment represents ^{32}P incorporated into the total protein fraction and is referred to as the 'washed' protein pellet.

Incubation of ganglia in the presence of octopamine did not

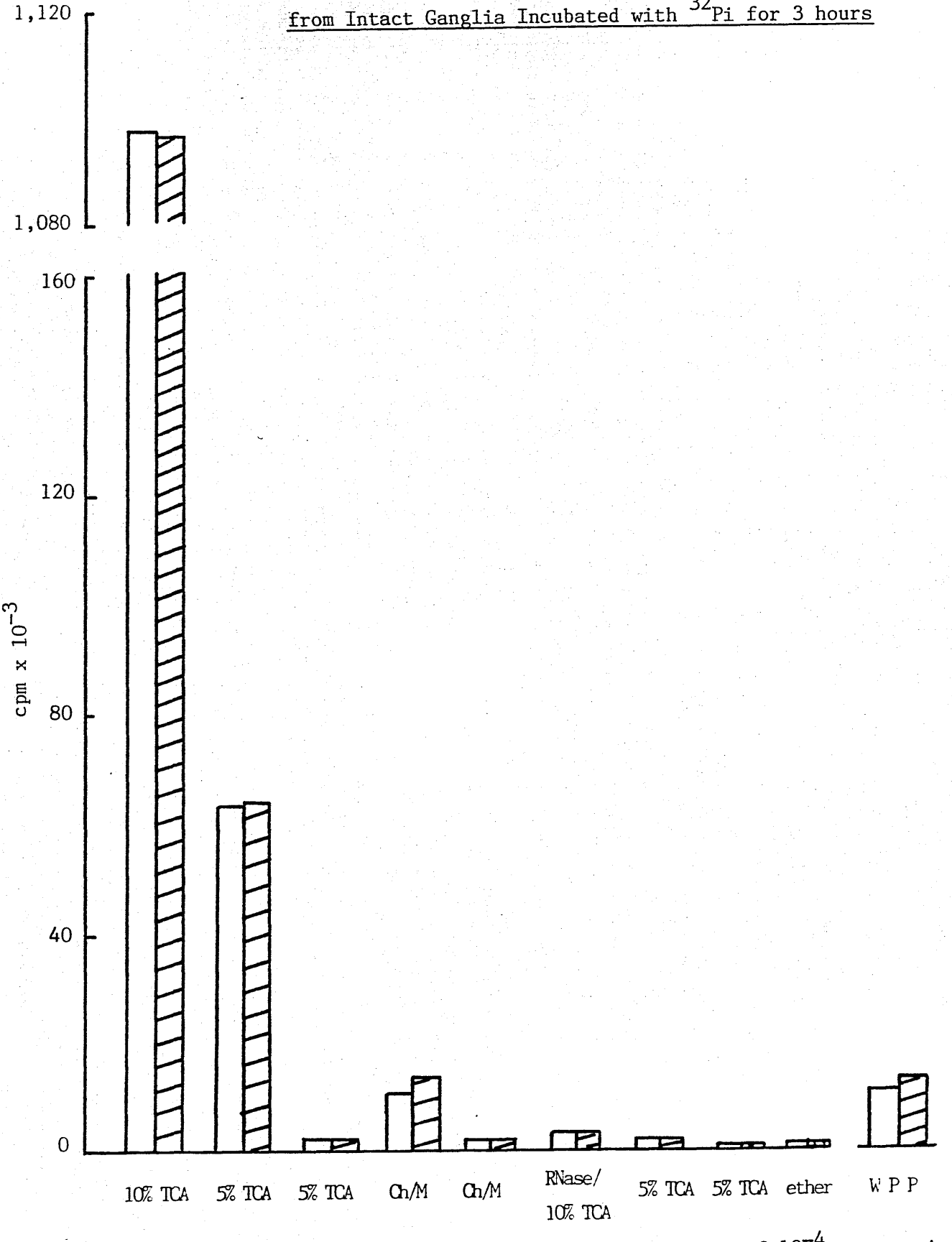
Figure: 13 Time Course of the Proportion of TCA Soluble and Insoluble Radioactivity



The proportion of TCA soluble (○) and insoluble (●) radioactivity from S. gregaria cerebral ganglia incubated with ^{32}Pi, homogenized with TCA and centrifuged at 10,000 g (Section 2:3:2).

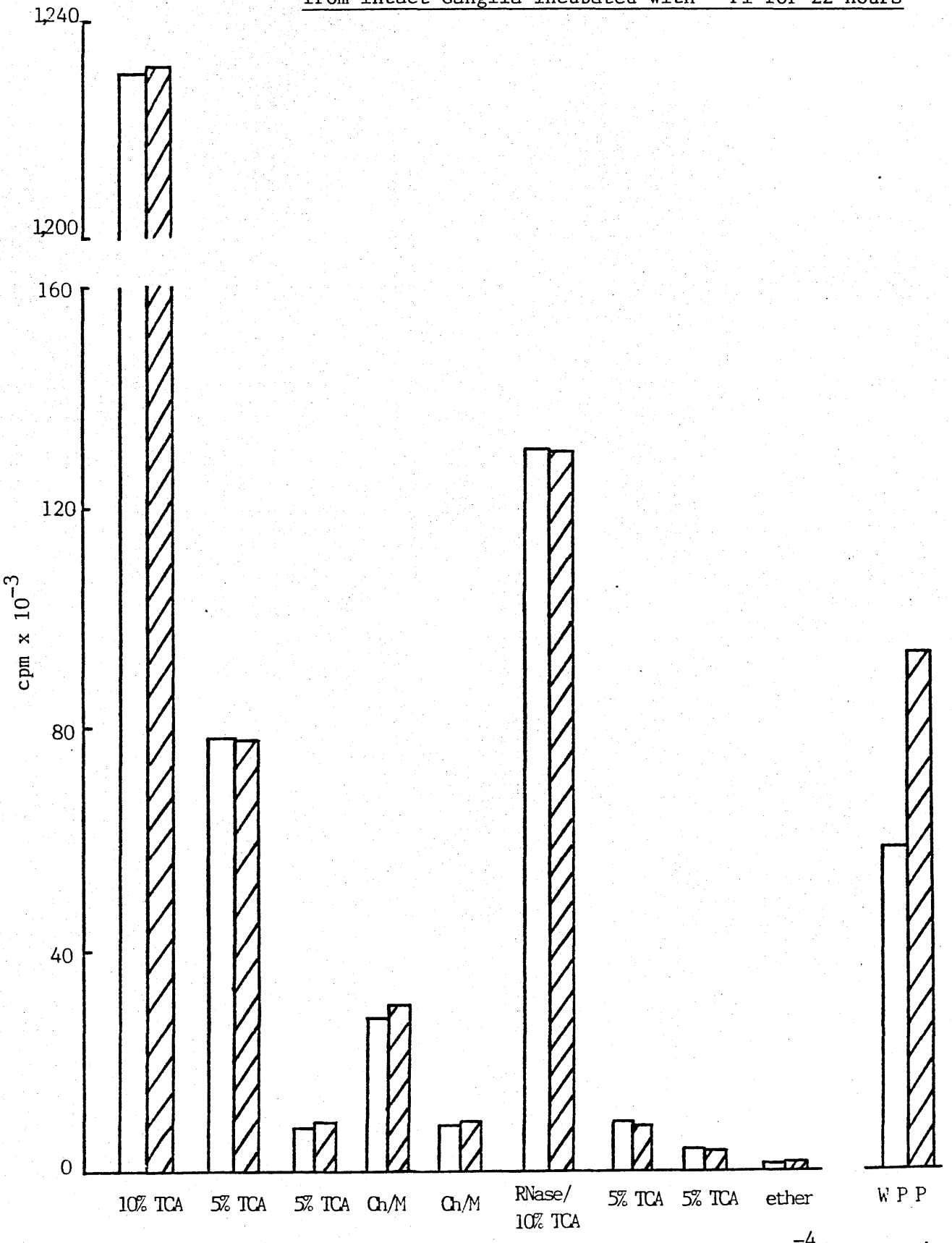
Results are the mean ± S.D. of 3 separate experiments.

Figure: 14 Fate of ^{32}P in the Preparation of 'Washed' Protein Pellets from Intact Ganglia Incubated with ^{32}Pi for 3 hours



Cerebral ganglia were incubated in the absence (▭) or presence of 10^{-4}M octopamine (▨) and the ^{32}P at each stage of the preparation (Section 2:3:2) of 'washed' protei pellets (WPP) was measured. Values shown are the means of duplicate measurements in one experiment and are typical of 3 separate experiments. Ch/M = chloroform/methanol.

Figure: 15 Fate of ^{32}P in the Preparation of 'Washed' Protein Pellets from Intact Ganglia Incubated with ^{32}Pi for 22 hours



Cerebral ganglia were incubated in the absence (▭) or presence of 10^{-4}M octopamine (▨) and the ^{32}P at each stage of the preparation (Section 2:3:2) of 'washed' protein pellets (WPP) was measured. Values shown are the means of duplicate measurements in one experiment and are typical of 7 separate experiments. Ch/M = chloroform/methanol.

appear to affect the amount of radioactivity removed at each stage of the extraction, however, more radioactivity remained in the washed protein pellets after 22 hours, (Figure 15).

Aliquots of the TCA soluble fraction from intact ganglia incubated with ^{32}Pi, (Section 2:4) were subjected to TLC on PEI cellulose sheets mainly to indicate the level of ^{32}P incorporated into ATP, (Figure 16 shows a typical separation of TCA soluble material).

Although the results were variable, (Table 6), in general, very little radioactivity was incorporated into ATP. There seemed to be a maximum incorporation between 12 and 18 hours, where between 5.6% and 10.4% of the total acid soluble ^{32}P recovered, migrated with ATP. In ganglia incubated for 1, 3 and 22 hours, less than 3% was incorporated into ATP. The radioactivity bound to GTP/GDP was much less than in ATP, $\leq$ 0.5% was detected. Most of the ^{32}P recovered from the acid soluble fraction was incorporated into ADP. This was only measured in one experiment, where between 1 and 22 hours, the proportion of ^{32}P incorporated into ADP remained between 60.4% and 72.3%. All of the remaining radioactivity on the TLC plates migrated with ^{32}Pi and AMP, (this combination of PEI cellulose/KH_2PO_4 did not resolve AMP from Pi, see Figure 16).

The total acid soluble radioactivity recovered in both experiments increased between 1 and 3 hours but decreased between 3 and 18 hours. The largest increase was observed in incubations between 18 and 22 hours for both experiments, (Table 6).

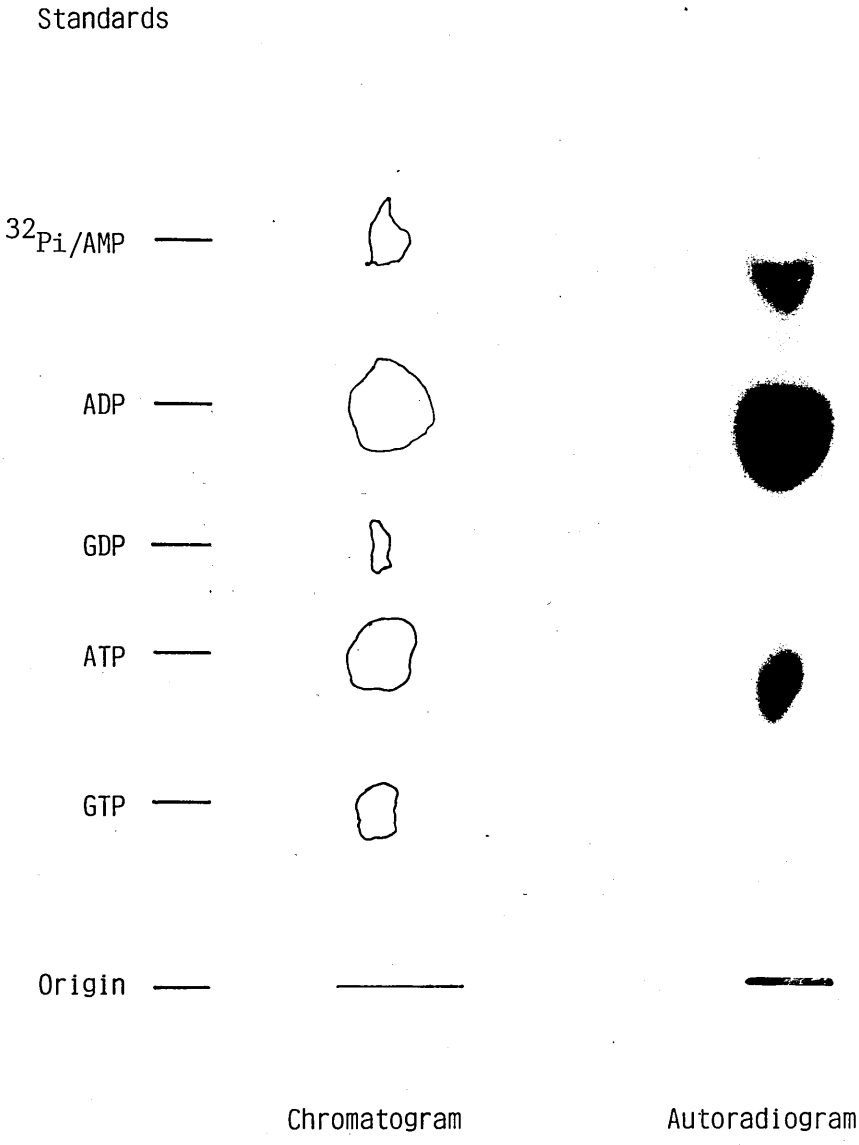


Figure: 16 Typical chromatography of TCA soluble material on PEI cellulose and the location of radioactivity. Cerebral ganglia were incubated with ^{32}Pi (section 2:3:1), homogenized in TCA and centrifuged (section 2:3:2). Aliquots of the acid soluble material containing the above standards were chromatographed and chromatograms autoradiographed (section 2:4).

Table: 6 Time Course of the Incorporation of ^{32}P into ATP, ADP and GTP/GDP from Intact Ganglia

Incubation conditions	Total TCA soluble ^{32}P recovered (cpm) Experiment 1	Experiment 2	% cpm in ATP Expt. 1	2	% cpm in ADP Expt. 1	2	% cpm in GTP/GDP Expt. 1	2
1 hour control	10,770	8,771	1.6	1.4	64.5	-	0.3	-
1 hour + OA	13,579	9,123	1.8	1.8	72.3	-	0.2	-
3 hours control	13,776	10,096	2.7	1.6	64.4	-	0.4	-
3 hours + OA	13,770	10,128	1.3	1.4	66.1	-	0.3	-
12 hours control	10,501	9,956	10.3	6.6	60.9	-	0.2	-
12 hours + OA	10,359	10,031	10.4	6.8	66.3	-	0.2	-
18 hours control	10,166	7,360	8.5	5.6	62.7	-	0.1	-
18 hours + OA	10,153	7,561	9.4	5.4	64.2	-	0.2	-
22 hours control	16,728	13,836	2.8	1.9	65.5	-	0.5	-
22 hours + OA	16,931	13,956	1.9	2.0	64.3	-	0.2	-

The TCA soluble fraction from intact cerebral ganglia which had been incubated with ^{32}Pi was prepared as described in section 2:4:1 and chromatographed on PEI cellulose (Section 2:4:2).
OA = octopamine ($10^{-4}M$) was included in incubations.

The presence of octopamine (10^{-4} M) in incubations of intact ganglia did not appear to have any reproducible effect on the incorporation of ^{32}P into ATP, ADP, GTP/GDP or into the total acid soluble fraction.

The incorporation of ^{32}P into ATP was measured in the presence of varying amounts of ^{32}Pi after 1 and 3 hours, (Table 7). As the ^{32}Pi concentration was increased from 1 µCi/ml to 6 µCi/ml, there was an increase in both the total acid soluble radioactivity and in the ^{32}P incorporated into ATP in each experiment. This occured to a greater extent in ganglia incubated for 3 hours than those incubated for 1 hour. ADP, GTP/GDP or the radioactivity migrating with AMP/^{32}Pi were not measured separately in these experiments.

Octopamine (10^{-4} M) did not have a reproducible effect on either the total acid soluble radioactivity or that incorporated into ATP under these conditions, (Table 7).

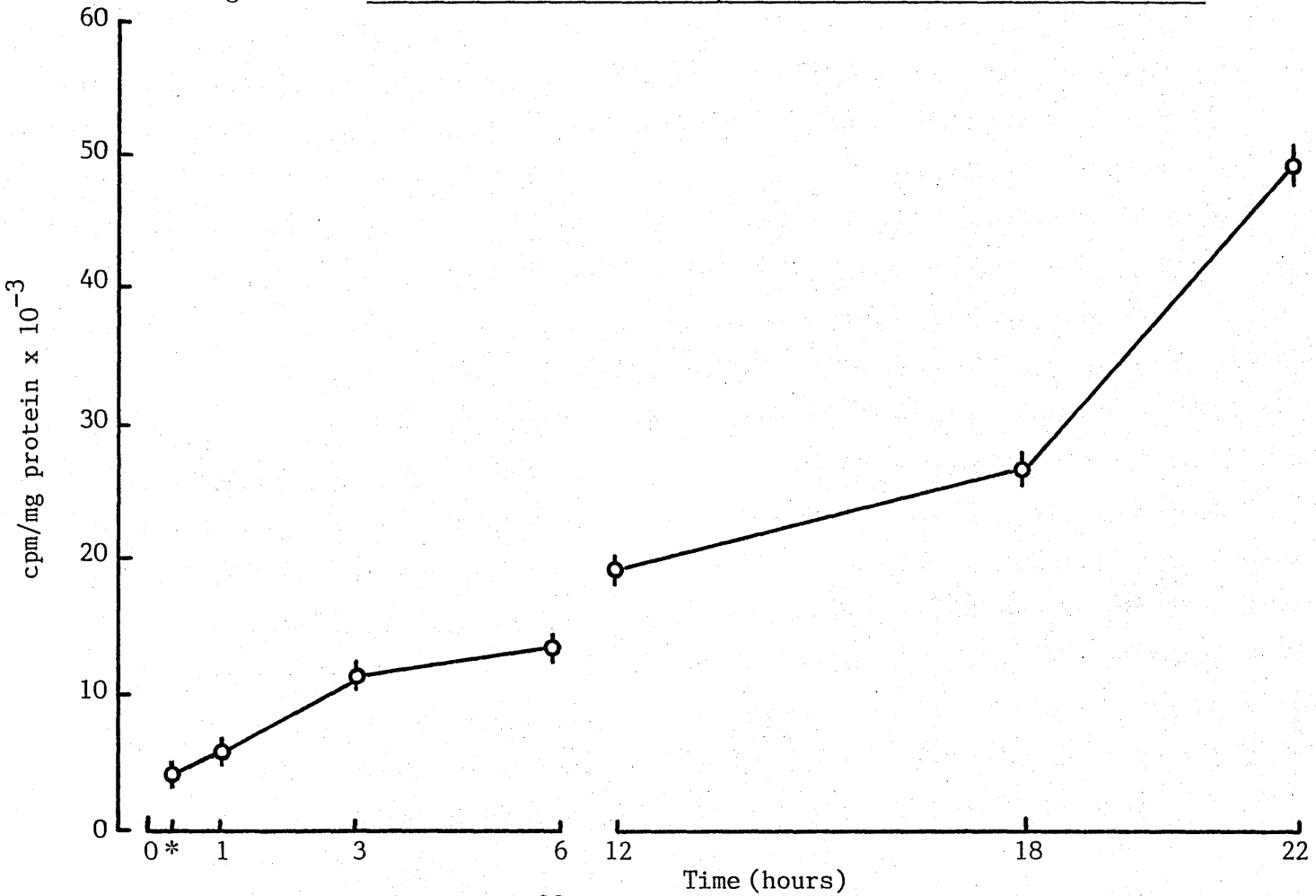
Incorporation of ^{32}P into the total protein fraction ('washed' protein pellets) from intact cerebral ganglia incubated with ^{32}Pi for up to 22 hours was also measured. Four separate experiments were carried out but these were not considered together as the specific activity of the ^{32}Pi varied from experiment to experiment. Figure 17 is representative of the time course observed in all 4 experiments although the absolute rates of ^{32}P incorporation described for Figure 17 were not observed in every experiment.

Table: 7 The Effect of Varying the Level of ^{32}Pi on the Incorporation of ^{32}P into ATP in Intact Ganglia

Time (hours)		^{32}Pi added (μCi/ml)	Total TCA soluble ^{32}P recovered (cpm) Expt. 1	Expt. 2	Expt. 3	^{32}P in ATP (cpm) Expt. 1	Expt. 2	Expt 3.
1	control	1	1,535	1,095	1,102	122	132	99
1	control	3	2,763	1,267	1,323	164	156	141
1	control	6	6,170	5,372	5,724	244	561	357
1	octopamine	1	2,483	1,347	1,346	197	107	121
1	octopamine	3	2,872	1,597	1,622	218	118	132
1	octopamine	6	8,702	4,397	2,655	394	693	289
3	control	1	1,881	1,672	1,570	146	176	112
3	control	3	6,668	2,123	1,869	556	178	111
3	control	6	17,667	7,895	2,303	1,430	335	291
3	octopamine	1	1,033	2,023	1,694	121	129	126
3	octopamine	3	7,016	3,568	1,992	498	212	145
3	octopamine	6	19,834	9,934	9,338	1,253	409	672

Intact cerebral ganglia were incubated with varying levels of ^{32}Pi in the absence (control) and presence of octopamine ($10^{-4}M$) for 1 and 3 hours. The TCA soluble fraction was then chromatographed on PEI cellulose (Section 2:4).

Figure: 17 Time Course of the Incorporation of ^{32}P into Total Protein



Intact cerebral ganglia were incubated with ^{32}Pi and the radioactivity incorporated into 'washed' protein pellets was measured (Section 2:3:2). Values shown are the means of duplicate measurements ± the range, and are typical of 4 separate experiments. *denotes 10 minute incubation.

Incorporation of ^{32}P into total protein occured in 3 phases, (Figure 17).

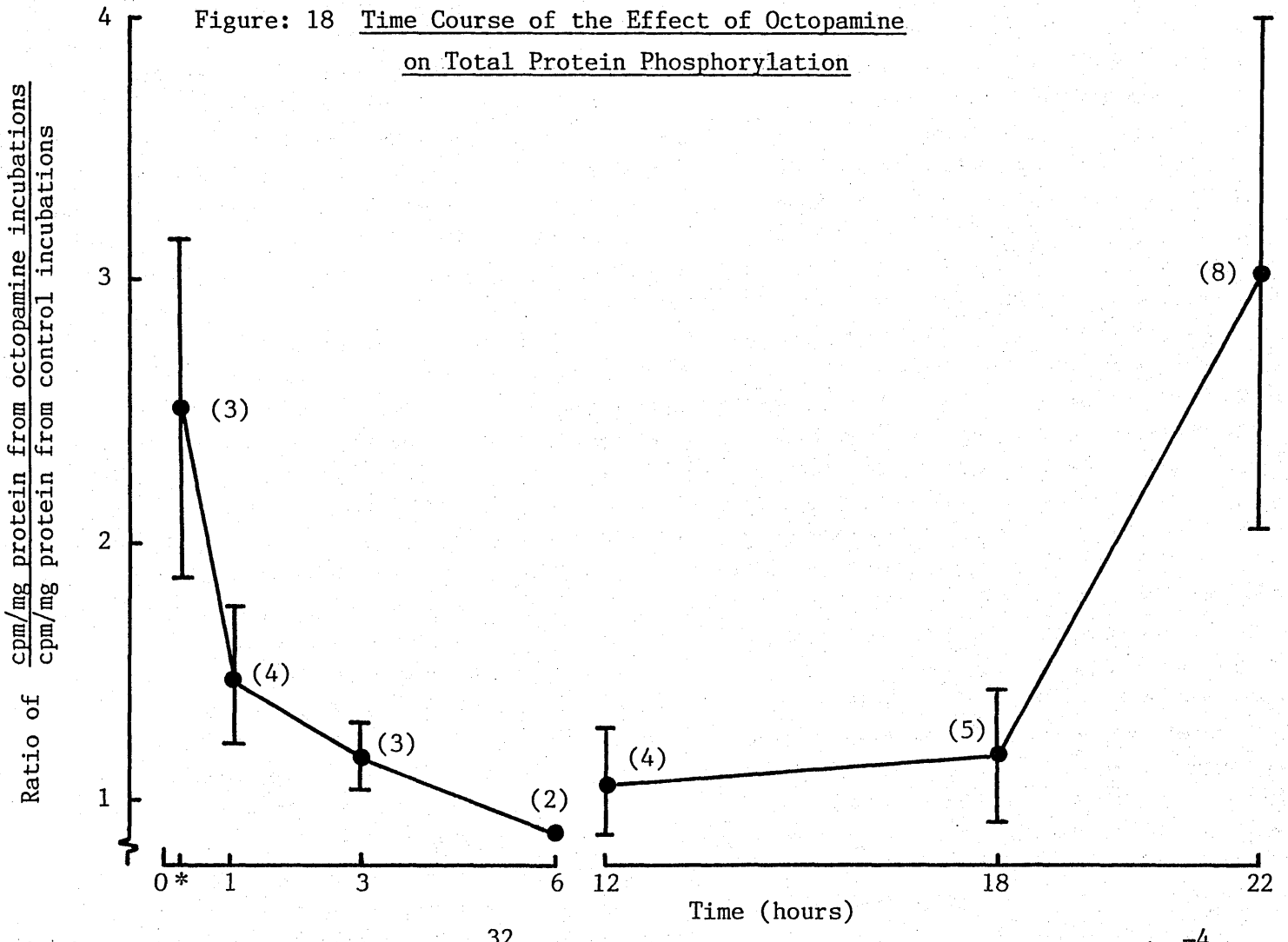
1) An initial linear increase between 10 minutes and 3 hours with a rate of 2,647 cpm/mg protein/hour.
2) A slower phase between 3 and 18 hours, where the rate decreased by more than one third to 833 cpm/mg protein/hour.
3) A rapid phase between 18 and 22 hours where the rate increased almost sevenfold to 5,625 cpm/mg protein/hour.

3:1:2 The Effect of Octopamine on the Phosphorylation of Total Protein

Octopamine (10^{-4} M) increased the incorporation of ^{32}P into total protein by a factor of 2.51 $\pm$ 0.65 in cerebral ganglia incubated for 10 minutes, (Figure 18). The magnitude of the increase declined to 1.2 $\pm$ 0.07 times the control value after 3 hours but no reproducible effect was observed between 6 and 18 hours. In 22 hour incubations, octopamine increased the total protein radioactivity for a second time by a factor of 3.0 $\pm$ 0.97.

N.B. All subsequent data presented in figures is representative of at least 3 separate experiments unless otherwise stated.

Figure: 18 Time Course of the Effect of Octopamine on Total Protein Phosphorylation



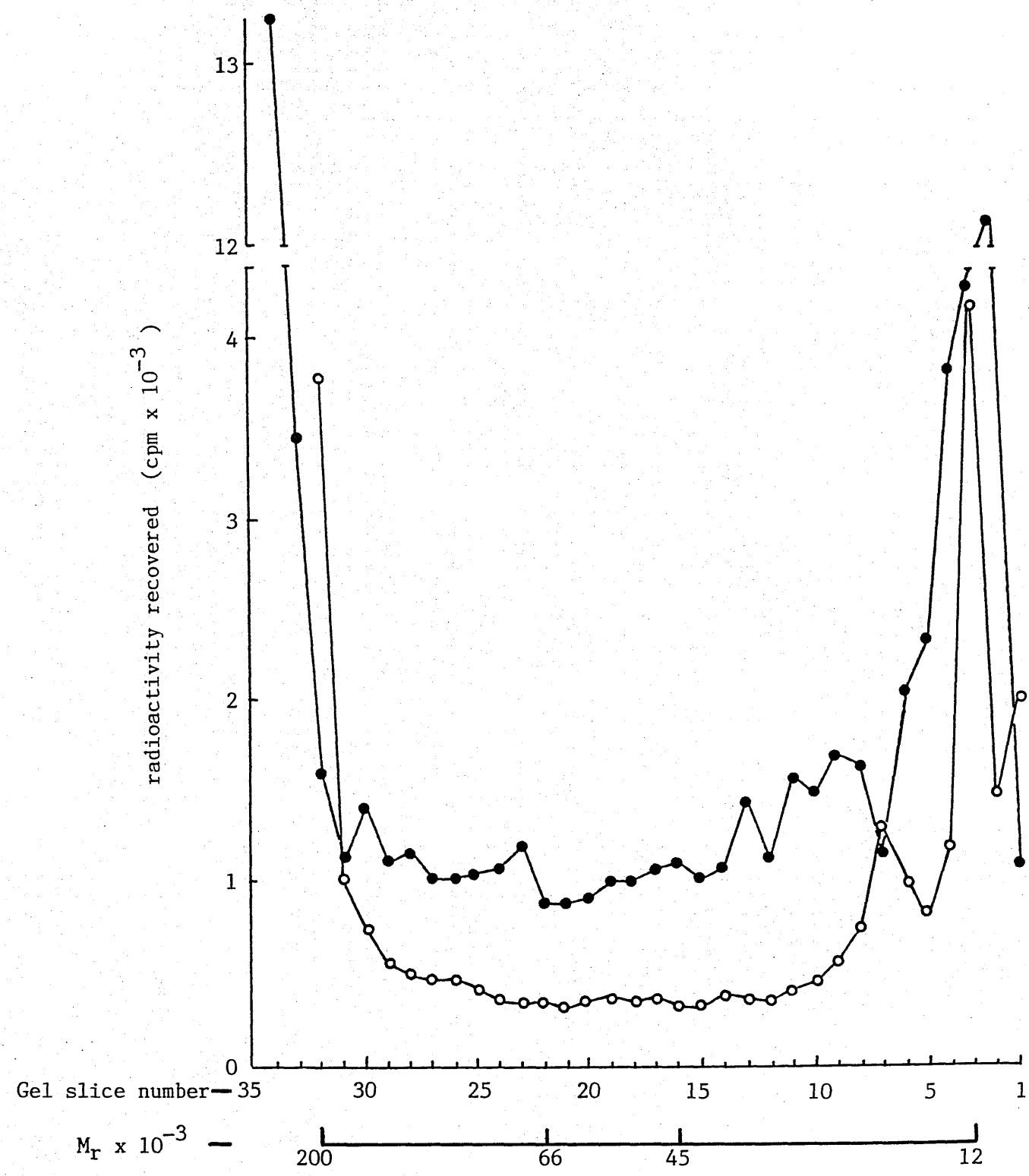
Intact cerebral ganglia were incubated with ^{32}Pi in the absence and presence of octopamine ($10^{-4}M$) and the ratio of ^{32}P in 'washed' protein pellets from octopamine preparations/control preparations was calculated for each experiment (Section 2:3:2). Values are the means ± standard deviations of the ratios. Numbers in brackets indicate the number of separate experiments. * denotes 10 minute incubations.

3:1:3 The Effect of Octopamine on the Phosphorylation of Specific Protein

Experiments in which the 'washed' protein pellets from ganglia incubated for 22 hours were subjected to rod-gel electrophoresis followed by slicing of the gels gave variable results. The radioactive profile contained many peaks which varied in intensity and position from one experiment to the next. This occured in both control and octopamine preparations. Figure 19 is only included as an example of this technique and is not representative of the other experiments (Figure 19 is Expt. 7,Table 8).

Similar unreliable gel patterns are obtained when Aplysia abdominal ganglia are incubated with radiolabelled phosphate for less than 15 hours, (Levitan et al, 1974). A disadvantage in the present study is using separate gels to compare the radioactivity patterns from control and octopamine incubated ganglia. In the study by Levitan et al, (1974) control and octopamine ganglia were incubated with ^{33}P and ^{32}P then combined for extraction and electrophoresis in the same gel, (each slice being counted for ^{33}P and ^{32}P). This obviates discrepancies (between gels) due to misalignment of the gel slices. In many experiments in the present study rod-gels prepared at the same time did not yield exactly the same number of slices. The volume of unpolymerized acrylamide at the interface of the SDS used to overlay the acrylamide solution (Section 2:3:3:2) varied from gel to gel. This would result in gels of slightly varying length.

Figure: 19 Rod-gel Electrophoresis of 'Washed' Protein Pellets



Intact cerebral ganglia were incubated with ^{32}Pi in the absence (O) and presence of 10^{-4}M octopamine (●) as described in Section 2:3:1. 'Washed' protein pellets were prepared (Section 2:3:2) and subjected to electrophoresis on 12.5% polyacrylamide/SDS rod-gels, (Section 2:3:3) and the radioactivity in each slice was measured. The pattern of radioactivity shown corresponds to experiment 7,(Table 8).

It was interesting to note that in 6 out of 7 experiments, (Table 8) the total radioactivity recovered from the gel was greater from octopamine incubations than from controls.

In order to overcome some of the problems associated with rod-gel electrophoresis, proteins were separated on slab-gels. This allowed samples to be compared on the same gel without the problem of alignment and dried gels could be autoradiographed, allowing the distribution of radioactivity to be visualized directly. Autoradiographs could be aligned with protein bands and consequently, quantitative measurements of the radioactivity, (by cutting out portions of the gel) could be carried out with greater accuracy than with individual rod-gels. In these experiments the ganglia homogenate was not subjected to the extraction procedure to remove phospholipids and RNA. Solubilizing buffer was added directly to the homogenates which were then boiled for 2 minutes. This allowed better resolution of the radioactive bands as preparations which were treated with TCA yielded diffuse bands. Slab-gels at the end of electrophoresis, were incubated in hot TCA, which has been shown to be an effective method of removing RNA from gels, (Bhorjee & Pederson, 1976).

The effect of octopamine was studied in preparations of intact ganglia which were incubated with octopamine for 10 minutes and 22 hours, as these two incubation times yielded the highest increases in total protein phosphorylation, (Figure 18).

Table: 8 Total Radioactivity recovered from Polyacrylamide rod-gels

Experiment	Addition to incubation	Total radioactivity recovered from gel (cpm)	Radioactivity added to gel (cpm)*
1	control	5,393	6,256
	octopamine	9,983	11,079
2	control	15,502	18,951
	octopamine	32,455	37,607
3	control	14,836	16,073
	octopamine	33,835	38,492
4	control	56,750	-
	octopamine	56,070	-
5	control	10,934	-
	octopamine	62,580	-
6	control	23,358	-
	octopamine	39,394	-
7	control	27,021	28,837
	octopamine	74,704	82,820

Intact cerebral ganglia were incubated with ^{32}Pi for 22 hours in the absence (control) and presence of octopamine ($10^{-4}M$). Ganglia were then homogenized and a 'washed' protein pellet was prepared, (Section 2:3) which was solubilized for electrophoresis on 12.5% polyacrylamide/SDS rod-gels, (Section 2:3:3). The total radioactivity recovered from each gel was the sum of the radioactivity present in each slice corrected to exclude background.

* occasionally aliquots of the solubilized 'washed' protein pellet (of the same volume as that placed on gels) were counted directly.

The effect of octopamine on specific protein phosphorylation after 10 minutes was studied by pre-incubating intact ganglia for 3 hours in the presence of a high level of ^{32}Pi (170 μCi/ml). Ganglia were pre-incubated with a high level of ^{32}Pi for two main reasons;

1) Preliminary experiments indicated that relatively little ^{32}P is incorporated into protein after only 10 minutes incubation, (Figure 17).
2) The incorporation of ^{32}P into ATP increased as the level of ^{32}Pi in the incubating medium was increased, (Table 7). A high level of radiolabelled ATP in the ganglia would be important as evidence obtained from mammalian nervous systems suggests that the phosphate group incorporated into protein is transferred from ATP, (Rodnight et al, 1975)

Ganglia were then placed in fresh iso-osmolar saline and incubated for 10 minutes in the absence or presence of either octopamine or dibutyryl cyclic AMP.

Addition of octopamine (10^{-4} M) to the incubation medium reproducibly stimulated the incorporation of ^{32}P into a single radioactive band with an apparent molecular weight of 39,000, (Figure 20). Gels were treated with hot TCA, so this radioactive band was unlikely to be RNA. Phospholipids, having low molecular weights, would migrate ahead of the tracking-dye in 10% polyacrylamide gels as do the phospholipids from mammalian nervous tissue, (Ehrlich et al, 1977). This indicates that octopamine stimulated the phosphorylation of an M_r 39,000 protein

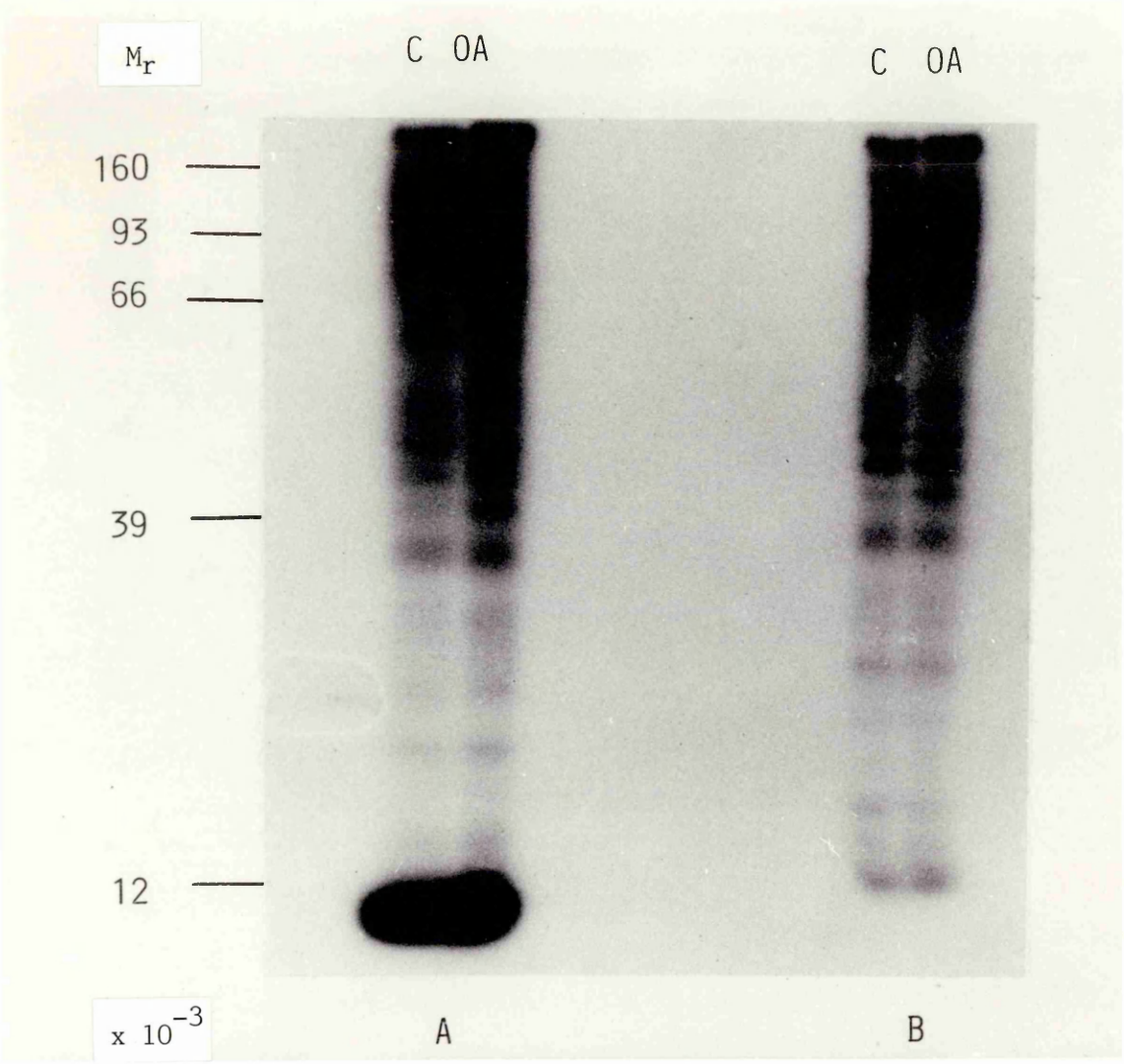


Figure: 20 The effect of octopamine on the phosphorylation of specific protein from intact ganglia after 10 minutes. Cerebral ganglia were pre-incubated with ^{32}Pi for 3 hours then incubated in the absence (C) or presence of 10^{-4} M octopamine (OA). Ganglia were then homogenized followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:3:4). Figure shows a comparison of autoradiographs from one half of a gel which had been treated with hot TCA (B) and the other half which was not treated, (A).

(or proteins). Figure 20 shows a comparison between one half of a gel which was treated with hot TCA and the other half stained as normal. TCA treatment did not affect the presence of radioactivity corresponding to M_r 39,000 in preparations from intact ganglia incubated with octopamine. Treatment of the gel with hot TCA did, however, remove all of the radioactivity migrating ahead of the tracking-dye.

Dibutyryl cyclic AMP (10^{-3} M) also greatly increased the incorporation of ^{32}P into an M_r 39,000 protein as well as stimulating an overall increase in phosphorylation, (Figure 21).

Incubation of intact ganglia with octopamine in the presence of 170 μCi/ml ^{32}Pi for 22 hours resulted in the increased incorporation of ^{32}P into an M_r 39,000 band as well as 2 other bands with apparent molecular weights fo 25,000 and 21,000, Figure 22). The effect of octopamine on the phosphorylation of these 3 bands was measured by cutting out the appropriate portions of the gel for liquid scintillation counting. The ratio of the cpm in octopamine preparations to the cpm in control preparations was calculated for those portions of the gel corresponding to molecular weights; 66,000 (which was not affected by octopamine) 39,000, 25,000 and 21,000. The mean $\pm$ standard deviations obtained for 3 separate experiments were 1.02 ± 0.09, 1.76 ± 0.38, 2.86 ± 0.91 and 1.82 ± 0.32 respectively.

The effect of octopamine on the phosphorylation of the M_r 39,000 protein after 22 hours could only be detected after treatment

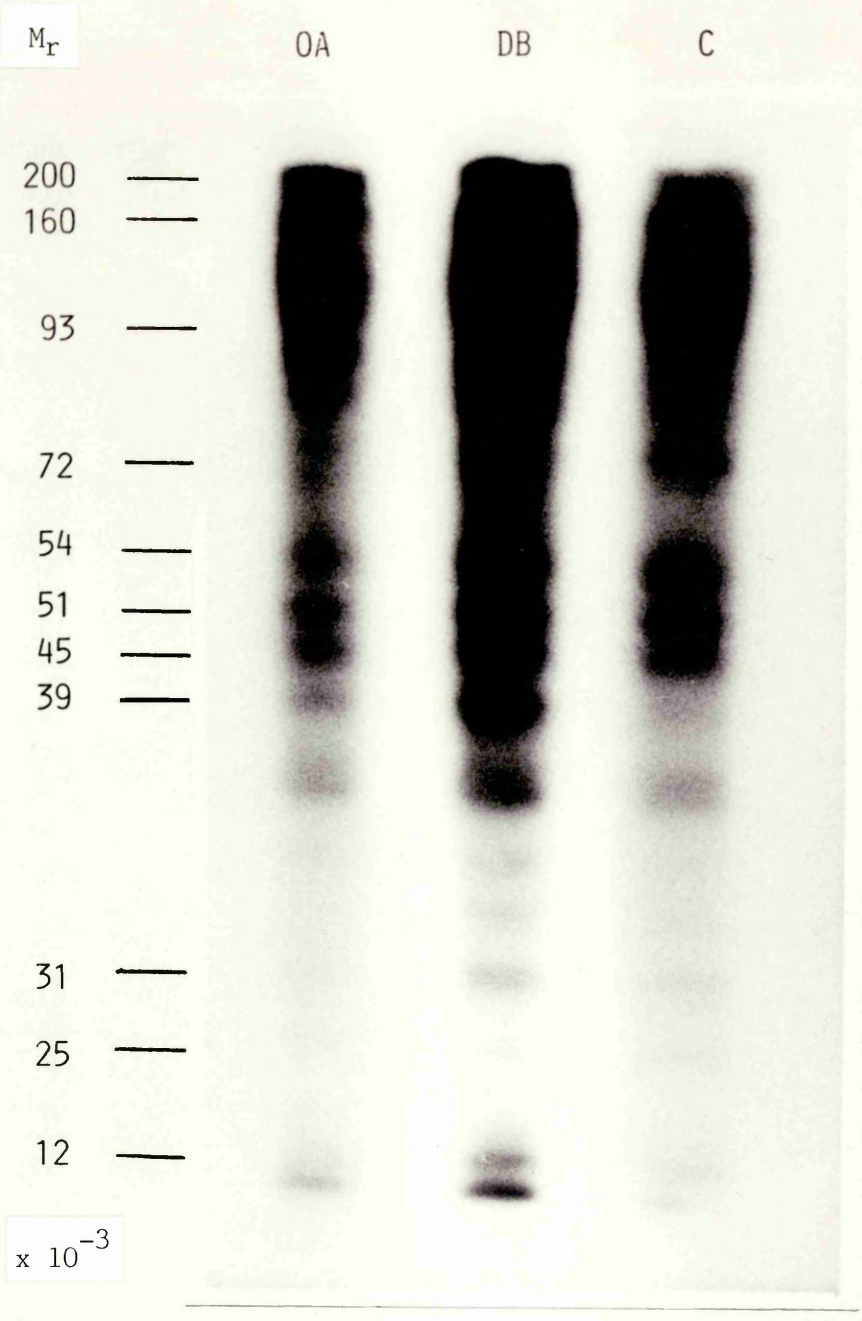


Figure: 21 The effect of dibutyryl cyclic AMP and octopamine on the phosphorylation of specific proteins from intact ganglia after 10 minutes. Cerebral ganglia were pre-incubated with ^{32}Pi then incubated in the absence (C) or presence of either 10^{-3} M dibutyryl cyclic AMP (DB) or 10^{-4} M octopamine (OA). Ganglia were then homogenized followed by electrophoresis on 10% polyacrylamide/ SDS gels which were then treated with hot TCA, (Section 2:3:4). Figure shows autoradiograph of gel.

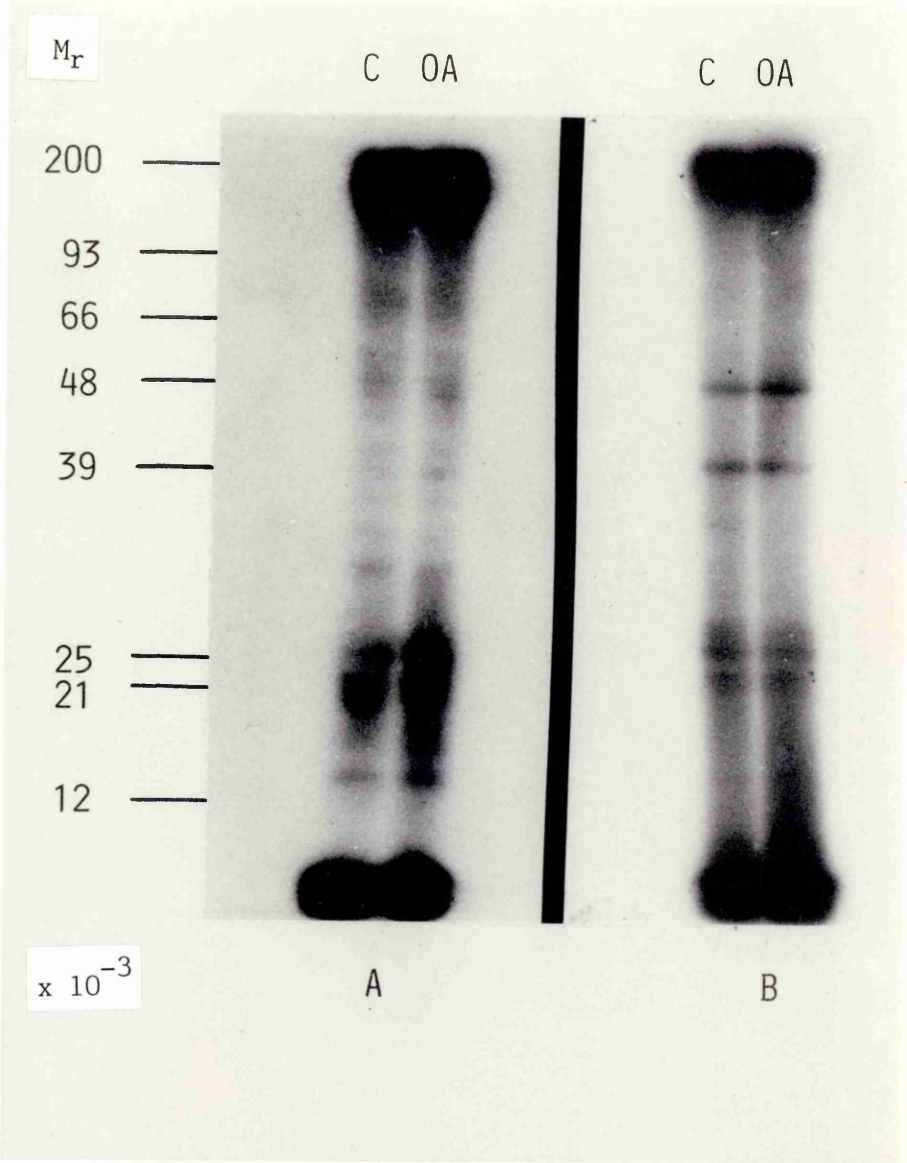


Figure: 22 The effect of octopamine on the phosphorylation of specific proteins from intact ganglia after 22 hours. Cerebral ganglia were incubated with ^{32}Pi in the absence (C) or presence of 10^{-4} M octopamine (OA) for 22 hours. Ganglia were then homogenized followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:3:4). Figure shows a comparison of autoradiographs from one half of a gel which was treated with hot TCA (A) and the other half which was not treated (B).

of the gels with hot TCA. This removed 2 intensely radioactive bands with apparent molecular weights of 40,000 and 48,000 in both control and octopamine preparations, (see Figure 22).

3:2 Protein Phosphorylation in Homogenized Tissue

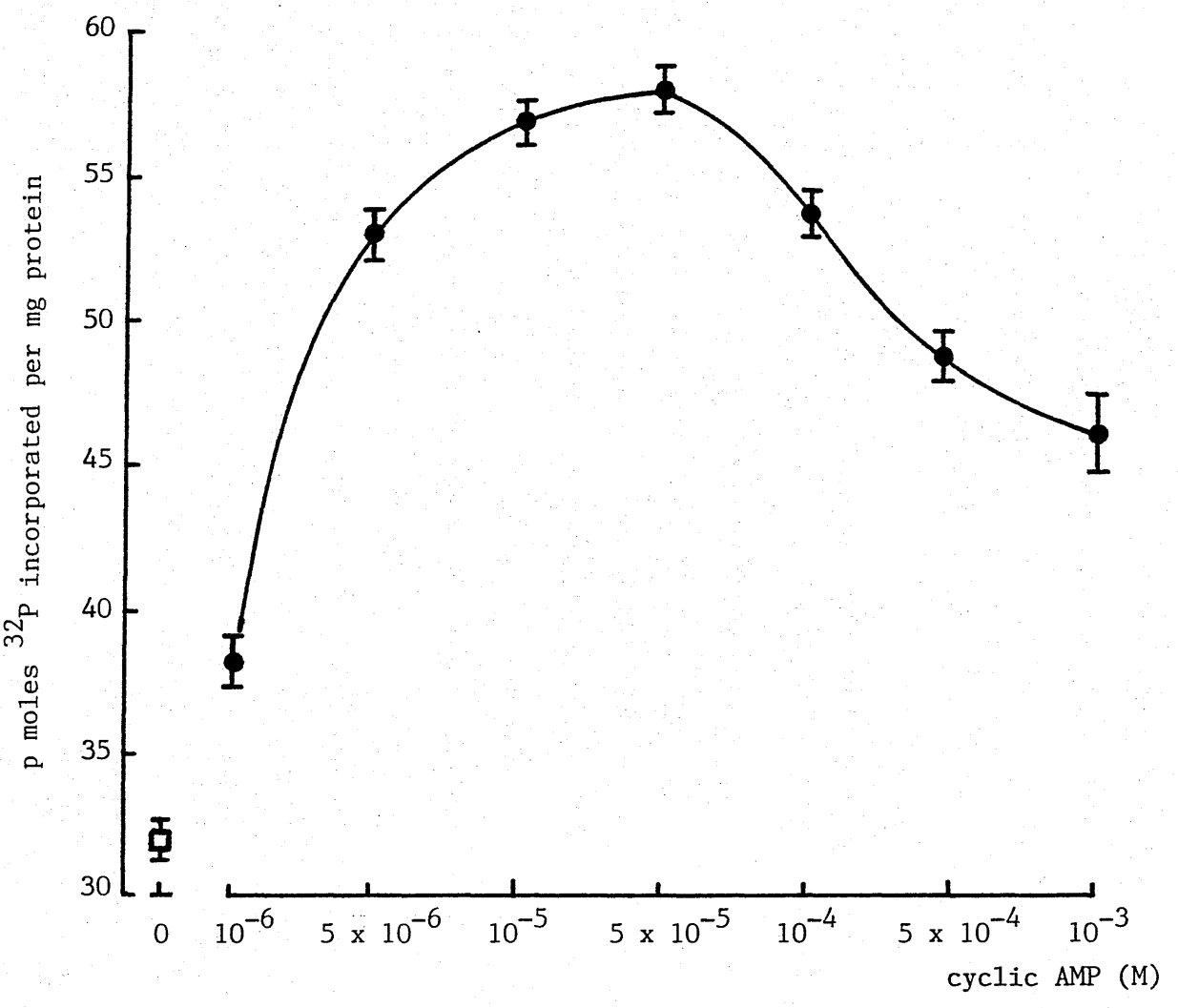
3:2:1 Effect of cyclic AMP on the Incorporation of Radioactivity into Total Protein

The effect of varying the concentration of cyclic AMP between 10^{-6} and 10^{-4} M on the phosphorylation of total protein was investigated. Cyclic AMP increased the incorporation of radioactivity into 'washed' protein pellets from homogenates of cerebral ganglia incubated with [γ^{32}P] ATP, (Figure 23). Maximum incorporation was achieved with 5 x 10^{-5} M cyclic AMP, higher concentrations resulted in a decrease.

3:2:2 The effect of cyclic AMP on the Radioactivity removed at each stage of extraction in the Preparation of 'Washed' Protein Pellets

Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the absence or presence of cyclic AMP (10^{-5} M). Incubations were terminated by adding TCA followed by centrifugation. The pellet obtained was extracted as described in section 2:3:2 and the radioactivity removed at each stage was measured, (Table 9). The major proportion of radioactivity was removed by the initial 10% TCA, although smaller amounts of radioactivity were removed by subsequent washes of the pellet with 5% TCA. Treatment of the pellet with chloroform/methanol removed a large proportion of the remaining radioactivity. The radioactivity removed by chloroform/methanol from cyclic AMP preparations was almost twice the amount removed in control preparations. This implies that cyclic AMP stimulated the incorporation of radioactivity from [γ^{32}P] ATP into chloroform/methanol soluble material, possibly phospholipids. The amount of protein present in pellets

Figure: 23 The Effect of Cyclic AMP on Total Protein Phosphorylation



Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the presence of varying concentrations of cyclic AMP and the radioactivity present in 'washed' protein pellets was measured (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

□ denotes incubations without cyclic AMP.

Table: 9 Fate of ^{32}P in the preparation of 'washed' protein pellets from homogenates incubated with [$\gamma^{32}P$] ATP

Homogenates of cerebral ganglia were incubated with [$\gamma^{32}P$] ATP for 1 minute (Secti 2:5:2) in the absence and presence of cyclic AMP (10^{-5}M). The radioactivity remove at each stage in the preparation of 'washed' protein pellets was then measured.

Treatment	control preparations (cpm)	Cyclic AMP preparations (cpm)	Protein in pellet (µg)
Initial 10% TCA	2·23 x 10^6 ± 5·4%	2·38 x 10^6 ± 6·8%	116 ± 4
1st 5% TCA wash	36,830 ± 2,940	33,131 ± 2,082	-
2nd 5% TCA wash	6,056 ± 1,839	8,256 ± 2,331	-
3rd 5% TCA wash	560 ± 125	880 ± 221	109 ± 4
chloroform/methanol 1st wash	7,190 ± 931	23,117 ± 1,924	-
chloroform/methanol 2nd wash	326 ± 84	944 ± 183	81 ± 4
10% TCA after RNase	460 ± 81	489 ± 65	-
1st 5% TCA wash	82 ± 10	95 ± 13	-
2nd 5% TCA wash	39 ± 5	43 ± 8	-
ether wash	41 ± 6	40 ± 8	-
washed protein pellets at the end of the above extraction	19,921 ± 2,850	64,560 ± 4,158	75 ± 4

Radioactivity measurements are the means ± standard deviations from 6 incubations. Protein measurements are the means of duplicate aliqouts (Section 2:7) ± the range

was also measured at various stages and this revealed that, of the protein present before the chloroform/methanol treatment, 74.1% remained in pellets after completion of the chloroform/methanol washes. This indicates that the other 25.9% protein was removed with the chloroform/methanol, as in previous experiments it was observed that cyclic AMP did not affect the protein present in the 'washed' pellets. The values obtained for incubations carried out and extracted as in Table 9 were 85 $\pm$ 3 µg protein for control incubations and 84 ± 4 µg protein in cyclic AMP (10^{-5} M) incubations, (values are means ± standard deviations for 5 separate incubations).

Resuspended delipidized pellets were incubated with ribonuclease followed by the addition of TCA and centrifugation. Very little radioactivity was present in the supernatant (TCA), i.e. very little radioactivity was removed from delipidized pellets by ribonuclease.

The pellets obtained after ribonuclease treatment were washed twice with 5% TCA and once with ether which removed very little additional radioactivity.

3:2:3 Pronase Treatment of Washed Protein Pellets

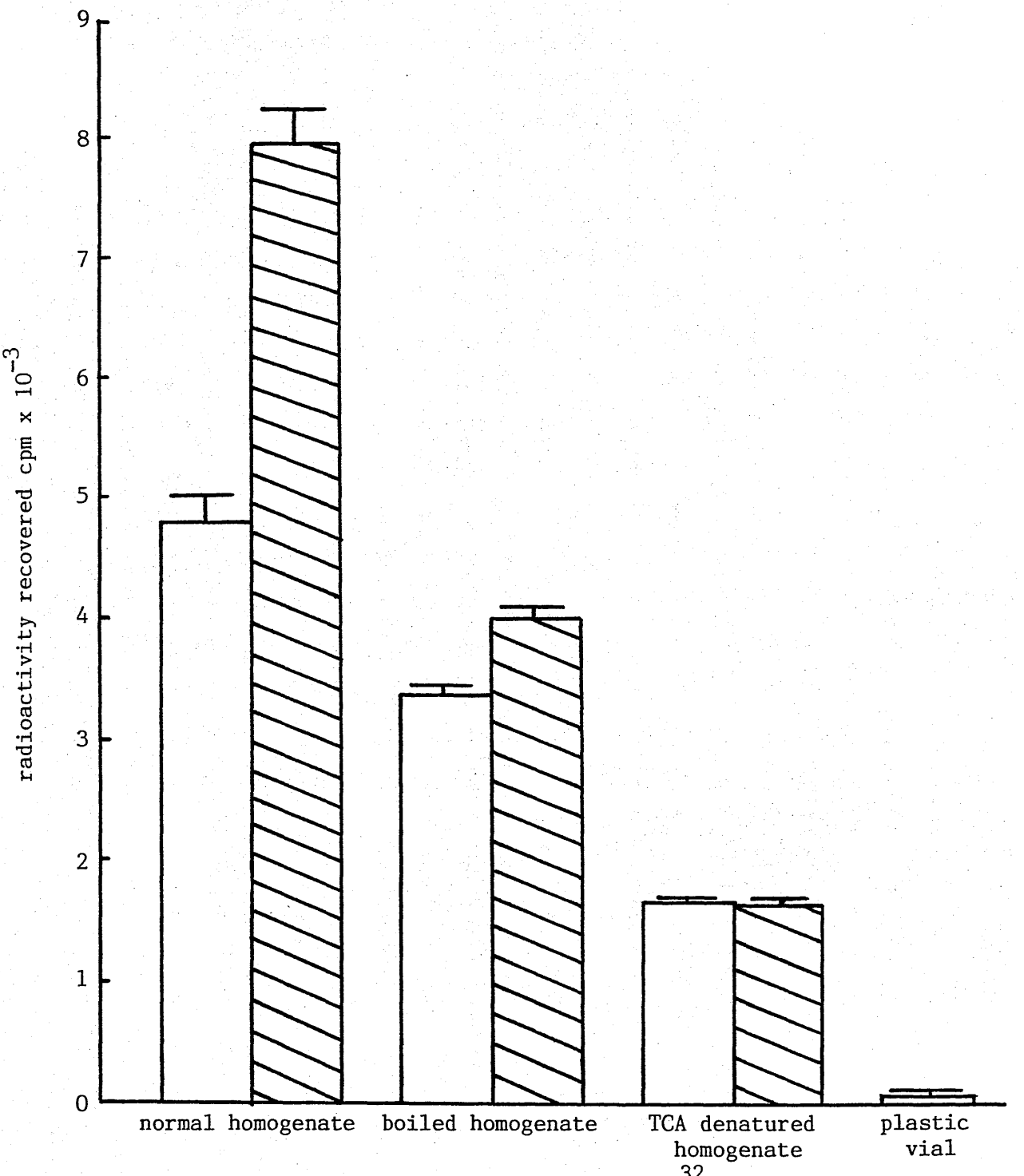
The washed protein pellets obtained at the end of extraction (Sections, 2:5:2/2:3) were resuspended in phosphate buffer and incubated in the presence or absence of pronase (Section 2:5:5). After 24 hours the incubation was terminated by adding TCA followed by centrifugation. Of the total radioactivity from washed protein pellets incubated without pronase 12.4% $\pm$ 0.5 remained in the supernatant (TCA) from control preparations and 11.2% $\pm$ 0.9 from cyclic AMP preparations, (Results are the means $\pm$ standard deviations of 3 separate incubations). The percentage of the total radioactivity from washed protein pellets which was rendered soluble in TCA by pronase treatment was 94.1% $\pm$ 1.2 from control preparations and 95.3% $\pm$ 0.3 from cyclic AMP preparations.

It was concluded from these results that almost all of the radioactivity present in washed protein pellets was associated with protein.

3:2:4 The Incorporation of Radioactivity into Total Protein from Homogenates which were Boiled or Treated with TCA

The incorporation of radioactivity into washed protein pellets from normal incubations were compared to those in which the incubation mixture containing homogenate was either boiled for 3 minutes or an equal volume of 10% TCA was added prior to adding [γ^{32}P] ATP. The radioactivity present in 'washed' protein pellets from homogenates which had been boiled prior to incubation with [γ^{32}P] ATP was less than that present in pellets from normal incubations, (Figure 24). Boiling did not

Figure: 24 Incorporation of ^{32}P into Total Protein from Boiled and TCA Denatured Homogenate Incubated with [$\gamma^{32}P$] ATP



Homogenates of cerebral ganglia were incubated with [$\gamma^{32}P$] ATP in the absence (▭) and presence of 10^{-5}M cyclic AMP (▨) as normal (Section 2:5:2) or the incubation mixture containing homogenate was either boiled for 3 minutes and allowed to cool for 5 minutes (boiled homogenate) or TCA was added for 5 minutes (TCA denatured) prior to adding [$\gamma^{32}P$] ATP. Incubations were also carried out in which homogenate was ommitted (plastic vial). All incubations were then treated for preparation of 'washed' protein pellets and the radioactivity recovered was measured. Values are means ± standard deviations from 3 separate incubations.

prevent an increased incorporation in the presence of cyclic AMP, although the percentage stimulation was reduced in comparison to the normal incubation. This suggests that boiling for 3 minutes did not adequately stop the reaction.

The addition of TCA to the incubation mixture prior to incubation with radiolabel resulted in a much lower incorporation of radioactivity than that in normal or "boiled" incubations. It also prevented the stimulation by cyclic AMP. This implies that the radioactivity associated with protein in 'washed' protein pellets from incubations pre-treated with TCA is not bound enzymically and possibly not covalently bound to protein. Very little radioactivity was recovered when incubations were carried out without homogenate in the incubation mixture. This indicated that very little of the radioactivity present in 'washed' protein pellets from normal incubations was due to radioactivity binding to the plastic vials and being released on solubilization of the protein, (Section 2:5:2).

These results suggested that the radioactivity associated with the protein present in the 'washed' pellets was not all enzymically bound. As much as 35.1% ± 0.6 was non-enzymically bound (the radioactivity present in pellets from homogenates pre-incubated with TCA) and was possibly adsorbed. It also appears that cyclic AMP stimulated an increase in the ^{32}P enzymatically bound to protein.

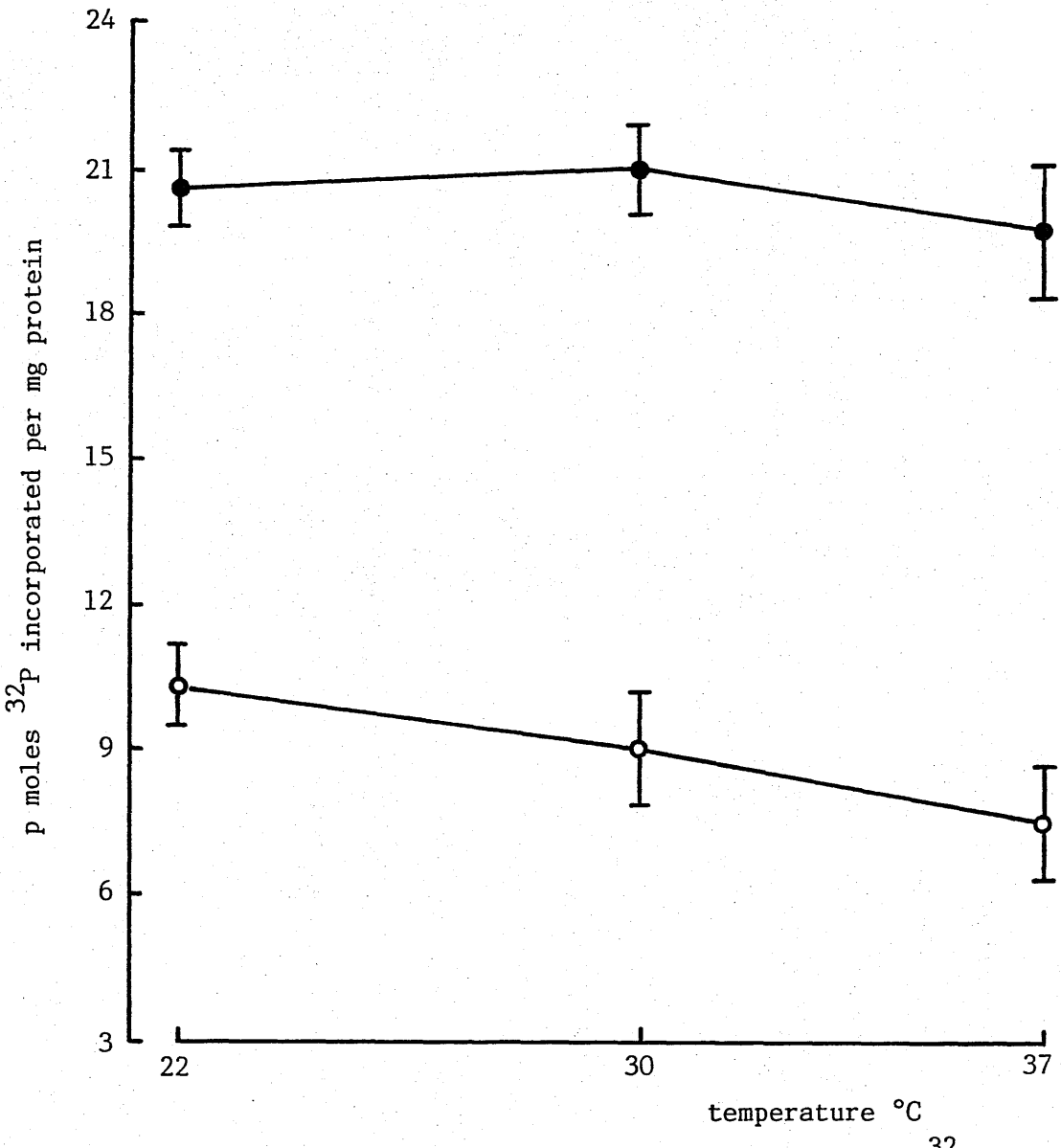
3:2:5 The Effect of Varying Incubation Conditions in the presence of Cyclic AMP on the Phosphorylation of Total Protein

Homogenates were incubated with [γ^{32}P] ATP at 22 °C, 33 °C and 37 °C. Varying the temperature of incubation within this range did not have any significant effect on the level of radioactivity incorporated into 'washed' protein pellets from control or cyclic AMP preparations, (Figure 25).

Homogenates were incubated with [γ^{32}P] ATP in buffers of varying pH in the range 4 to 8. The incorporation of radioactivity into 'washed' protein pellets from both control and cyclic AMP preparations increased with increasing pH. A maximum incorporation was achieved at pH 7. Increasing the pH to 8 resulted in a decrease, (Figure 26).

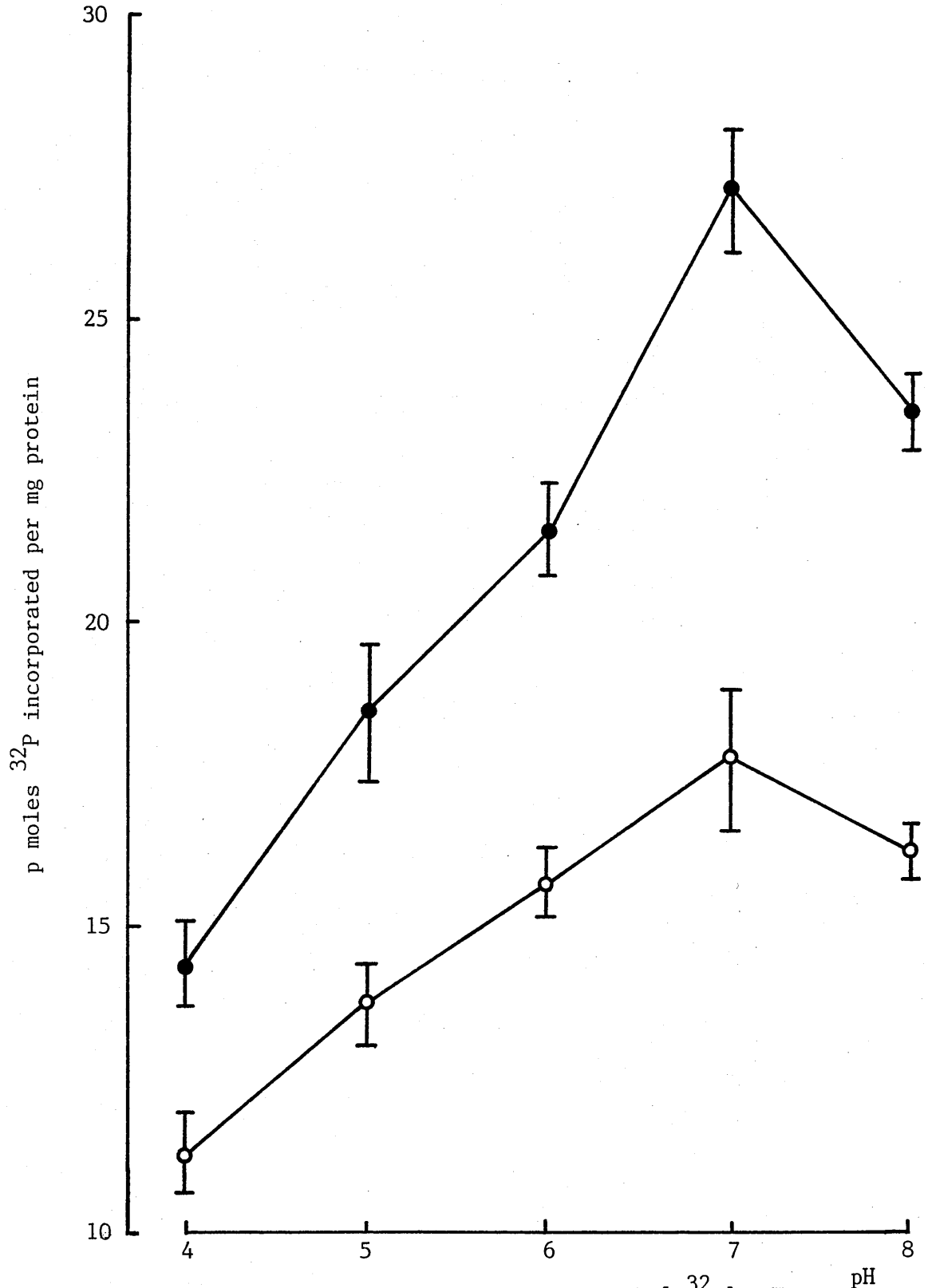
Homogenates were incubated with 0.5, 1.6 and 2.6 µCi [γ^{32}P] ATP in the presence and absence of cyclic AMP. As the level of [γ^{32}P] ATP in the incubating medium was increased the radioactivity present in 'washed' protein pellets also increased in direct proportion. This occured in both control and cyclic AMP preparations. The difference between the radioactivity present in control and cyclic AMP preparations also increased in proportion to the level of [γ^{32}P] ATP, (Figure 27). The actual incorporation of ^{32}P corrected for the change in specific activity, however, remained constant. The values obtained for incubations with 0.5, 1.6 and 2.6 µCi were; 2.18 ± 0.71, 1.99 ± 0.27, 2.41 ± 0.71 p moles per mg protein respectively in control preparations and 5.09 ± 1.09, 4.49 ± 0.27, 4.58 ± 0.23 respectively in cyclic AMP preparations (each value is the mean ± standard deviation of 3 separate incubations)

Figure: 25 The Effect of Temperature on Total Protein Phosphorylation



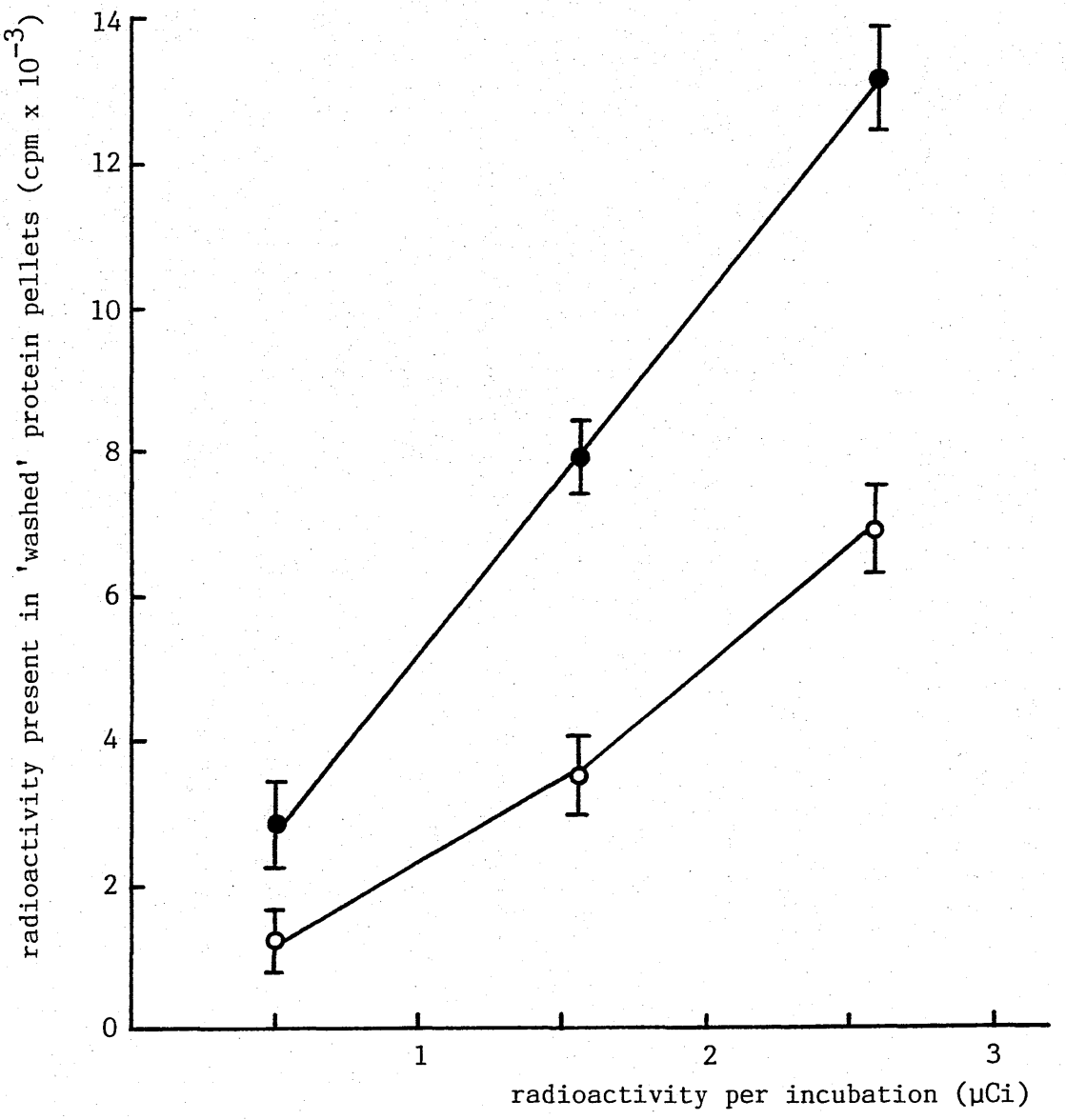
Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the absence (O) or presence of 10^{-5}M cyclic AMP (●) at various temperatures and the radioactivity present in 'washed' protein pellets was measured (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

Figure: 26 The Effect of pH on Total Protein Phosphorylation



Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP at varying pH in the absence (O) and presence of 10^{-5}M cyclic AMP (●) and the radioactivity present in 'washed' protein pellets was measured, (Section 2:5:2). pH 4, 5 and 6 were achieved with Na_3 citrate/citric acid (60 mM) and pH 6 and 7 with the normal buffer Tris/maleate (80 mM). Values are the means ± standard deviations from 3 separate incubations.

Figure: 27 The Effect of Varying the Amount of [γ^{32}P] ATP on the Phosphorylation of Total Protein

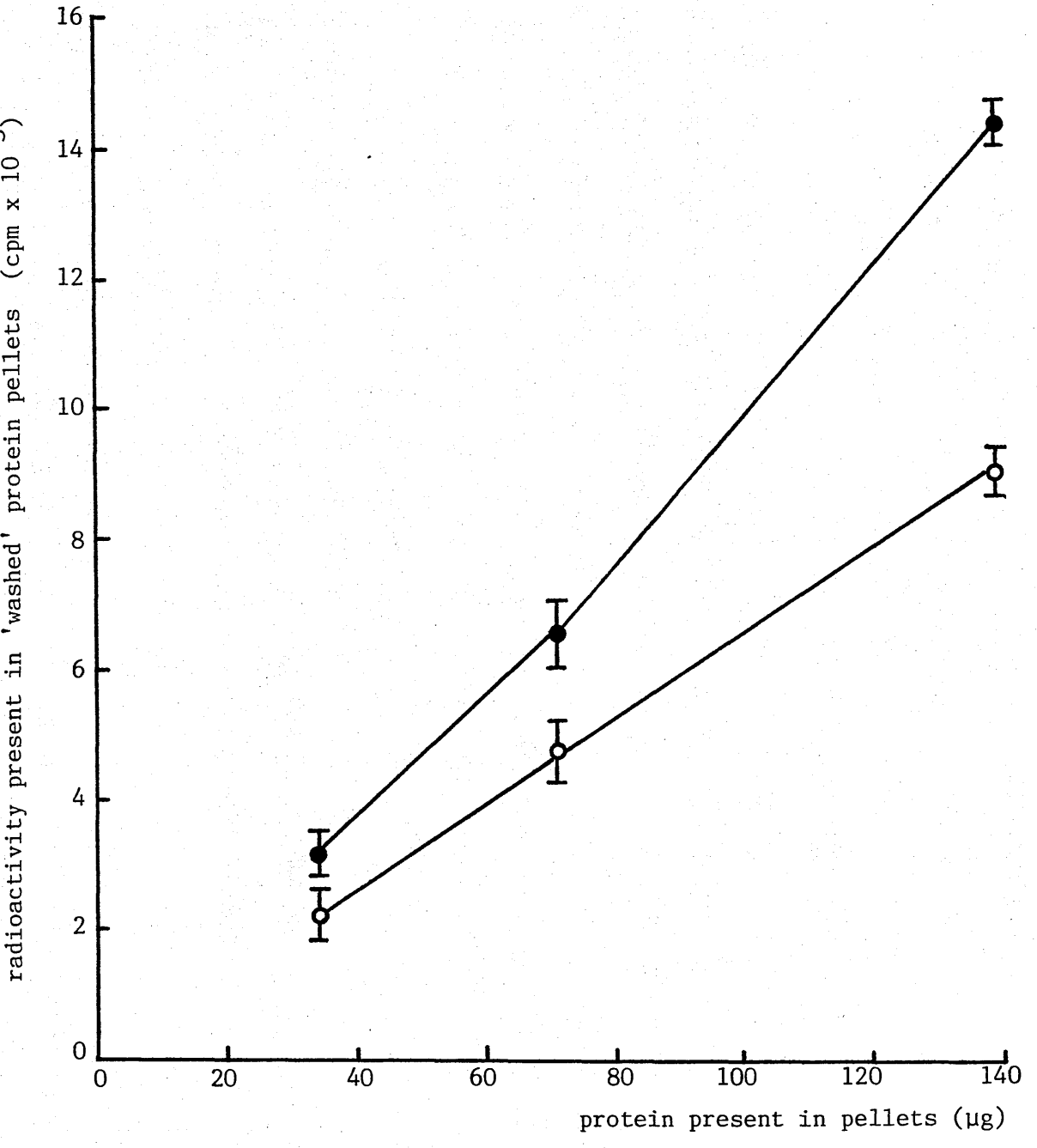


Homogenates of cerebral ganglia were incubated with varying amounts of [γ^{32}P] ATP in the absence (O) and presence of 10^{-5}M cyclic AMP (●) and the radioactivity present in 'washed' protein pellets was measured (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

Homogenates with varying concentrations of tissue (30, 60 and 120 mg/ml) were incubated with [γ^{32}P] ATP, in the presence or absence of cyclic AMP. Incorporation of radioactivity into 'washed' protein pellets was proportional to the amount of protein (in homogenates) added. This occured in both control and cyclic AMP preparations, (Figure 28).

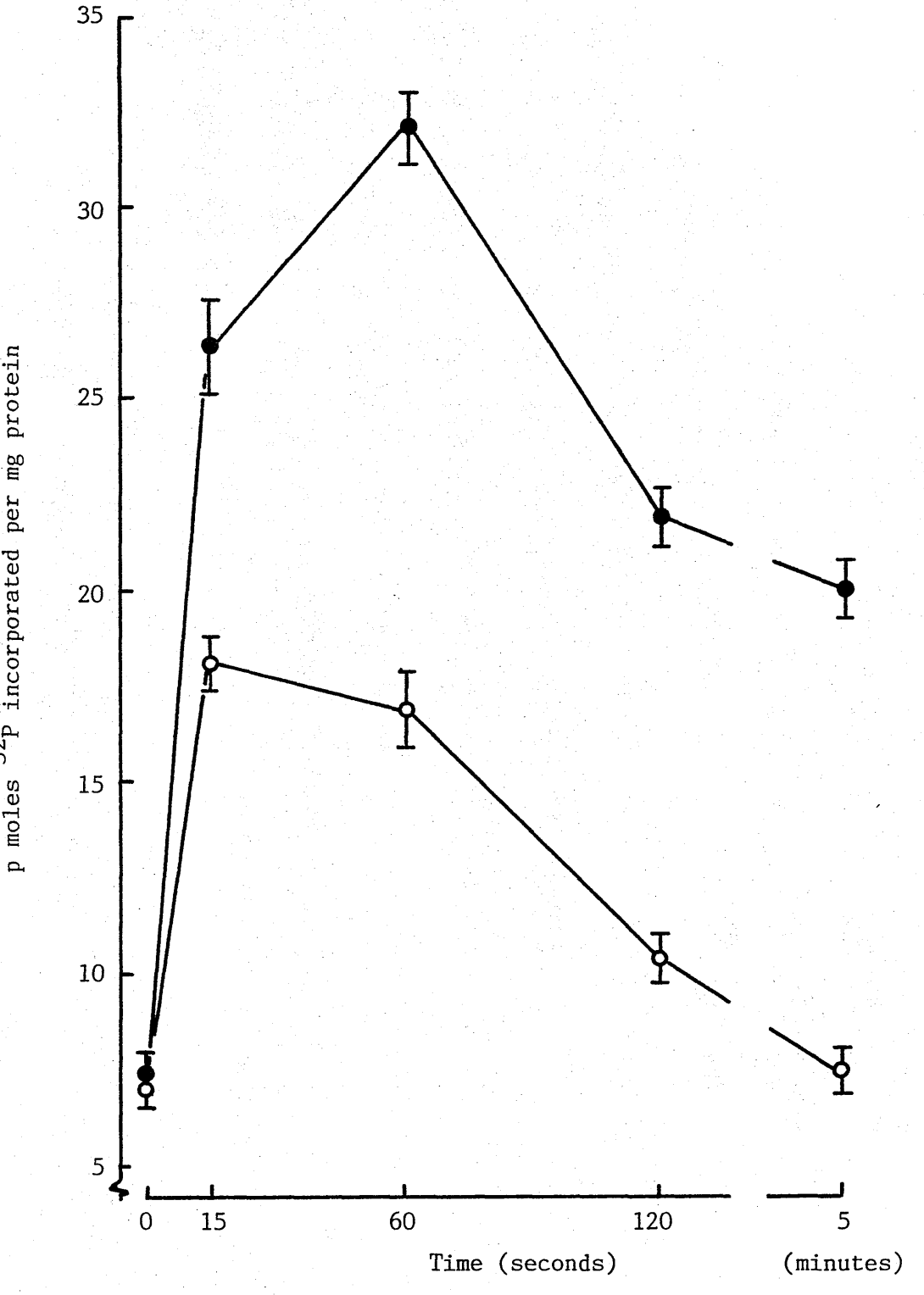
The time course of incorporation of radioactivity into 'washed' protein pellets from homogenates incubated with [γ^{32}P] ATP, was studied over a 5 minute period. A maximum incorporation of radioactivity into 'washed' protein pellets from control preparations was achieved after 15 seconds, (Figure 29). This decreased to the zero-time level within 5 minutes. In contrast, the incorporation of radioactivity into 'washed' protein pellets from cyclic AMP preparations although higher than control preparations after 15 seconds, did not reach a maximum level until 1 minute. The greatest difference in incorporation between control and cyclic AMP preparations was also observed after 1 minute. The level of radioactivity was still higher in cyclic AMP preparations after 5 minutes than in control preparations.

Figure: 28 The Effect of Varying the Amount of Homogenate on the Phosphorylation of Total Protein



Homogenates with varying concentrations of tissue (30, 60 and 120 mg/ml) were incubated with [γ^{32}P] ATP in the absence (O) and presence of 10^{-5}M cyclic AMP (●) and the radioactivity present in 'washed' protein pellets was measured (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

Figure: 29 Time Course of the Phosphorylation of Total Protein



Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP for various times in the absence (o) or presence of 10^{-5}M cyclic AMP (●) and the radioactivity present in 'washed' protein pellets was measured. Values are the means ± standard deviations from 3 separate incubations.

3:2:6 The Effect of Octopamine on the Phosphorylation of Total Protein

The effect of octopamine on the phosphorylation of total protein was studied under the same conditions as those in which a cyclic AMP stimulated increase was observed. Octopamine (10^{-4} M) did not significantly alter the level of radioactivity present in washed protein pellets under these conditions, (Table 10). Octopamine stimulates a 3.4 fold increase in the level of cyclic AMP under similar conditions in the presence of GTP (10^{-4} M) and 500 μM ATP, (Kilpatrick et al, 1980). The effect of octopamine on the phosphorylation of total protein was therefore studied in homogenates which were incubated with [γ^{32}P] ATP in the presence of GTP with 10 μM ATP and 500 μM ATP. No significant effect of octopamine on the level of radioactivity in washed protein pellets could be detected under these conditions, (Tables 10 and 11).

Substituting GppNHp for GTP also had no significant effect on the incorporation of radioactivity in the presence of octopamine, (Table 11).

The effect of varying the concentration of unlabelled ATP between 10 μM and 500 μM on the phosphorylation of total protein was investigated. Increasing the concentration of ATP increased the incorporation of radioactivity into washed protein pellets in a curvilinear manner, (Figure 30). Incorporation into pellets from cyclic AMP preparations was significantly greater than control preparations for each concentration of ATP up to 200 μM. No significant difference was observed between cyclic AMP and

Table: 10 The Effect of Octopamine on Total Protein Phosphorylation (10μM ATP)

Additions to incubations	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Buffer only	15·1 ± 0·4	50·4 ± 3·3	19·2 ± 1·2	96·8 ± 2·6
Buffer + GTP	-	-	19·6 ± 1·4	95·3 ± 4·2
Octopamine	16·0 ± 0·7	51·7 ± 2·8	-	-
Octopamine + GTP	-	-	17·9 ± 2·8	94·2 ± 4·8
Cyclic AMP	54·3 ± 3·0**	92·1 ± 5·6**	-	-

Homogenates of cerebral ganglia were incubated as described in section 2:5:2 with the above additions; GTP (10^{-4}M), octopamine (10^{-4}M) and cyclic AMP (10^{-5}M), in the presence of [γ^{32}P] ATP. Values are the means of the p moles ^{32}P incorporate per mg protein ± standard deviations for 3 separate incubations.

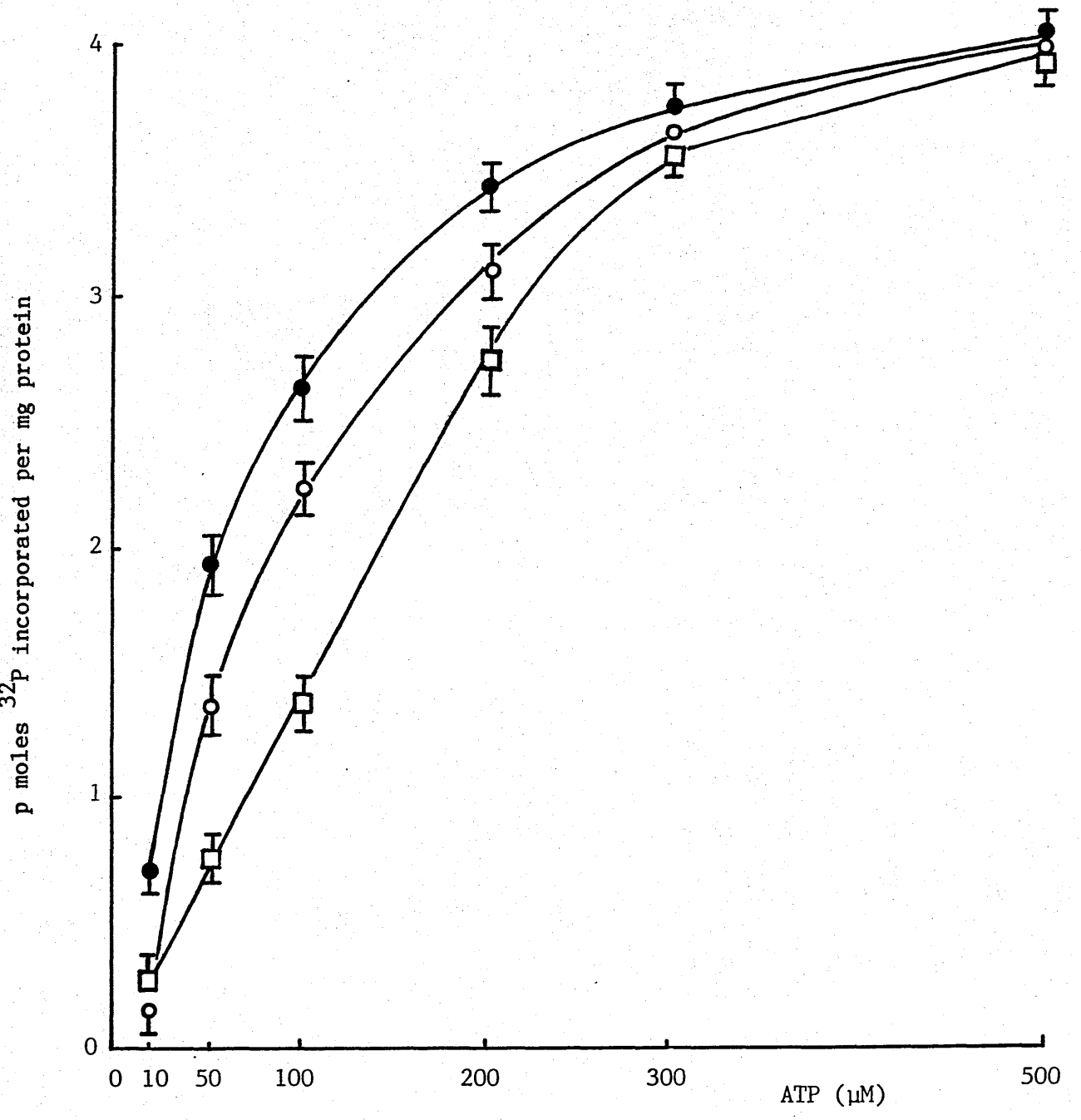
Table: 11 The Effect of Octopamine on Total Protein Phosphorylation in the presence of 500 μM ATP

Additions to incubations	Experiment 1	Experiment 2	Experiment 3	Experiment 4
Buffer + GTP	10·9 ± 1·8	17·6 ± 2·1	49·8 ± 2·6	8·3 ± 1·1
Octopamine + GTP	11·5 ± 1·4	19·2 ± 1·6	50·2 ± 1·9	-
Buffer + GppNHp	-	-	51·4 ± 2·8	8·9 ± 1·4
Octopamine + GppNHp	-	-	52·4 ± 2·3	9·1 ± 0·9

Homogenates of cerebral ganglia were incubated as described in section 2:5:2 with the above additions; GTP (10^{-4}M), GppNHp (10^{-4}M) and octopamine (10^{-4}M) in the presence of 500 μM ATP containing [γ^{32}P] ATP. Values are as described for Table 11.

**Significantly higher than control (buffer only), $P < 0{\cdot}005$.

Figure: 30 The Effect of varying the ATP concentration on Total Protein Phosphorylation in the presence of Octopamine and Cyclic AMP



Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the presence of either buffer alone (o), 10^{-5}M cyclic AMP (●) or 10^{-4}M octopamine (□) with varying concentrations of ATP. The radioactivity present in 'washed' protein pellets was then measured, (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

control preparations with ATP concentrations of 300 μM and 500 μM.

Octopamine (10^{-4} M) did not significantly alter the level of radioactivity in washed protein pellets from incubations with 10 μM ATP. A significant decrease was, however, observed with ATP concentrations between 50 μM and 200 μM. No significant difference in incorporation was observed between 300 μM and 500 μM ATP.

Octopamine stimulates an increased synthesis of cyclic AMP in homogenates, (Kilpatrick et al, 1980) and a decrease in the incorporation of radioactivity into total protein under similar conditions, (Figure 30). This suggests that in incubations with octopamine the [γ^{32}P] ATP was being used up in 2 processes,

a) Cyclic AMP synthesis

b) Protein phosphorylation

The decrease in total protein phosphorylation is, therefore, probably due to a decrease in the [γ^{32}P] ATP available for protein phosphorylation.

An increase in total protein phosphorylation in the presence of octopamine could be detected if homogenates were pre-incubated for 5 minutes with unlabelled ATP (100 μM) and GTP before the addition of [γ^{32}P] ATP for one minute. Under these conditions a significant increase in the radioactivity present in protein

pellets from octopamine preparations was observed, (Table 12). Substituting GppNHp for GTP under these conditions results in a greater incorporation of radioactivity into total protein, both in the presence and absence of octopamine.

The radioactivity incorporated into total protein in the presence of octopamine was significantly reduced if sodium fluoride (10^{-2} M) was also present.

3:2:7 The Effect of Calcium on the phosphorylation of Total Protein

The effect of varying the Ca^{2+} concentration between 250 µM and 1,500 µM in the presence of EGTA (700 µM) on the phosphorylation of total protein was studied. Maximum incorporation was achieved with a $CaCl_2$ concentration of 1,000 µM,, higher concentrations resulted in a decrease, (Figure 31).

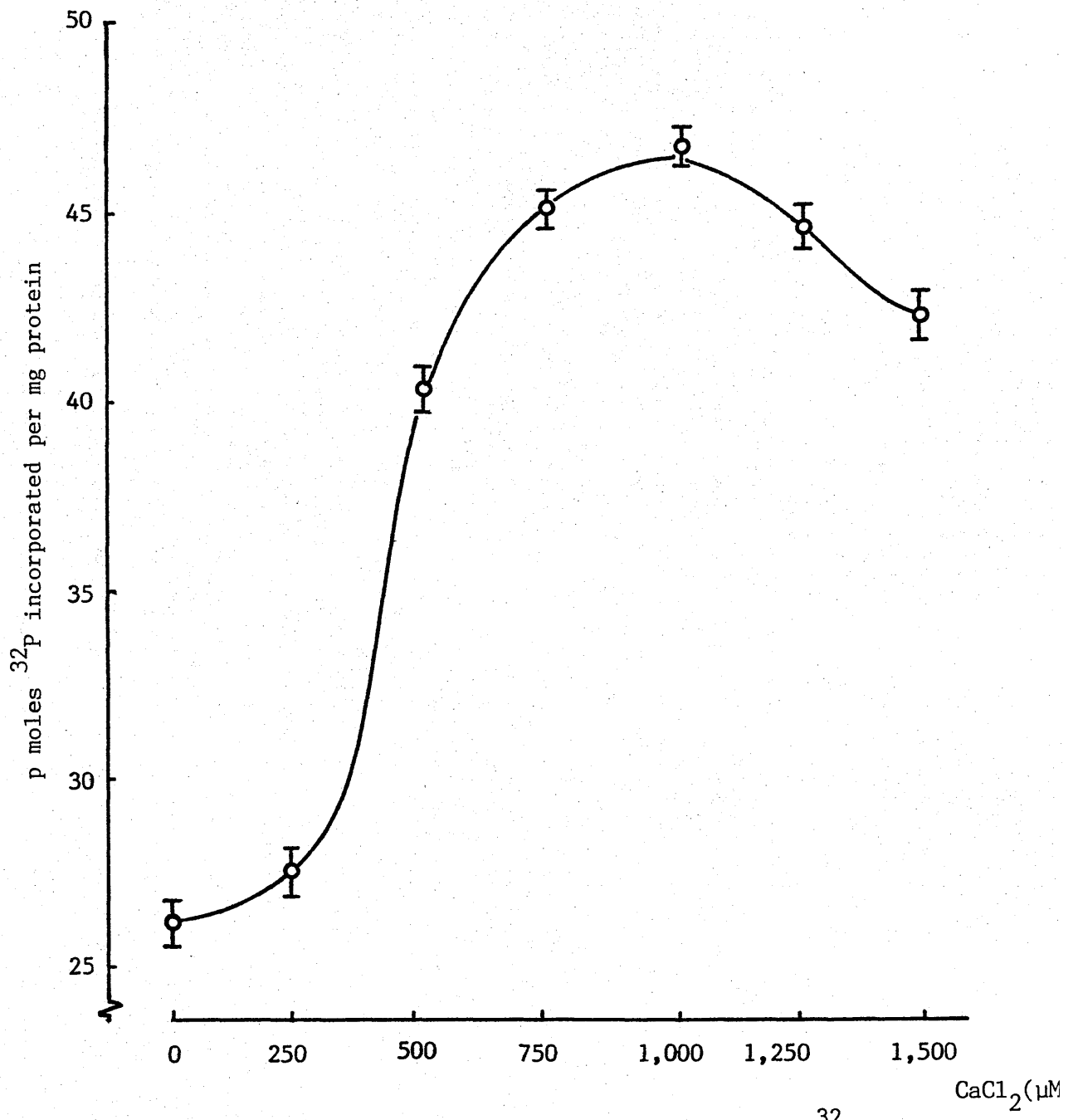
Table: 12 The Effect of Pre-incubating Tissue Homogenate with Unlabelled ATP before adding [γ^{32}P] ATP on Total Protein Phosphorylation

Additions to incubations	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
Buffer + GTP	38·8 ± 2·9	21·3 ± 1·8	53·5 ± 3·6	34·4 ± 2·8	7·3 ± 1·5
Octopamine + GTP	49·1 ± 4·3*	30·6 ± 4·8*	62·8 ± 3·8*	42·3 ± 2·3*	20·4 ± 1·1**
Buffer + GppNHp	–	–	59·8 ± 2·7	38·8 ± 2·2	–
Octopamine + GppNHp	–	–	75·9 ± 3·9*	47·7 ± 3·2*	–
NaF + GTP	–	–	–	–	6·4 ± 1·2
Octopamine + NaF + GTP	–	–	–	–	[1] 12·8 ± 2·9*

Homogenates of cerebral ganglia were incubated with the above additions; GTP (10^{-4}M), octopamine (10^{-4}M) GppNHp (10^{-4}M) and NaF (10^{-2}M) in the presence of 100 µM ATP for 5 minutes before the addition of [γ^{32}P] ATP for 1 minute. Radioactivity incorporated into total protein was then measured as described in section 2:5:2/2:3:2 . Values are the means of the p moles ^{32}P incorporated per mg protein ± standard deviations for 3 separate incubations.

*indicates probability of < 0·05 and **probability < 0·01 for t-tests against control (buffer + GTP) values. [1] Significantly lower than with octopamine + GTP alone P < 0·05.

Figure: 31 The Effect of Calcium on Total Protein Phosphorylation



Homogenates of cerebral ganglia were incubated with [γ^{32}P]ATP in the presence of varying concentrations of $CaCl_2$ and the radioactivity present in 'washed' protein pellets was measured,(Section 2:5:2). Values are the means ± standard deviations of 3 separate incubations.

EGTA (700 μM) was present in all incubations.

3:2:8 The Phosphorylation of Total Protein in Subcellular Fractions

3:2:8:1 Soluble (Sf) and Particulate (Pf) fractions

The Sf and Pf were prepared in hypoosmotic buffer as described in Section 2:5:1:1. Electron micrographs revealed that the Pf contained many membrane-like structures, (Figure 32), indicating that the Pf was a crude membrane fraction. No particulate material was visible by light microscopy when samples of the Sf were negatively stained. This indicated that the Sf was composed of soluble tissue material.

3:2:8:1a The Effect of Cyclic AMP

The incorporation of radioactivity from [γ^{32}P] ATP into total protein from Sf was greater than into total protein from crude homogenate. This occured in both control and cyclic AMP preparations, (Figure 33). Cyclic AMP (10^{-5} M) increased the incorporation above control in both fractions. Incorporation of radioactivity into total protein from Pf was lower than in total protein from either Sf or crude homogenate. Cyclic AMP, however, did not have a significant effect in the Pf.

Experiments were carried out in which the crude homogenate was incubated with [γ^{32}P] ATP in the presence and absence of cyclic AMP before preparation of Sf and Pf, (Section 2:5:4:1). Less radioactivity was present in total protein from the Sf and Pf than in the crude homogenate, under these conditions, (Figure 33). Significantly more radioactivity was present in total protein obtained from incubations with cyclic AMP for both Sf and Pf than from control incubations.

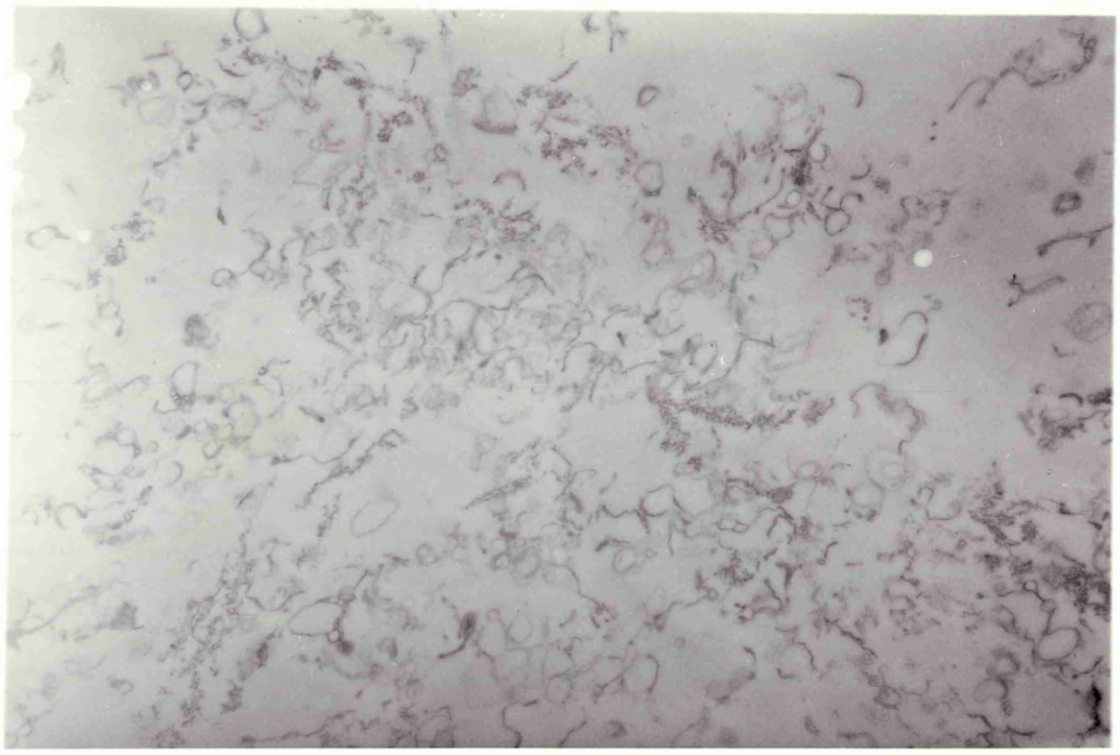
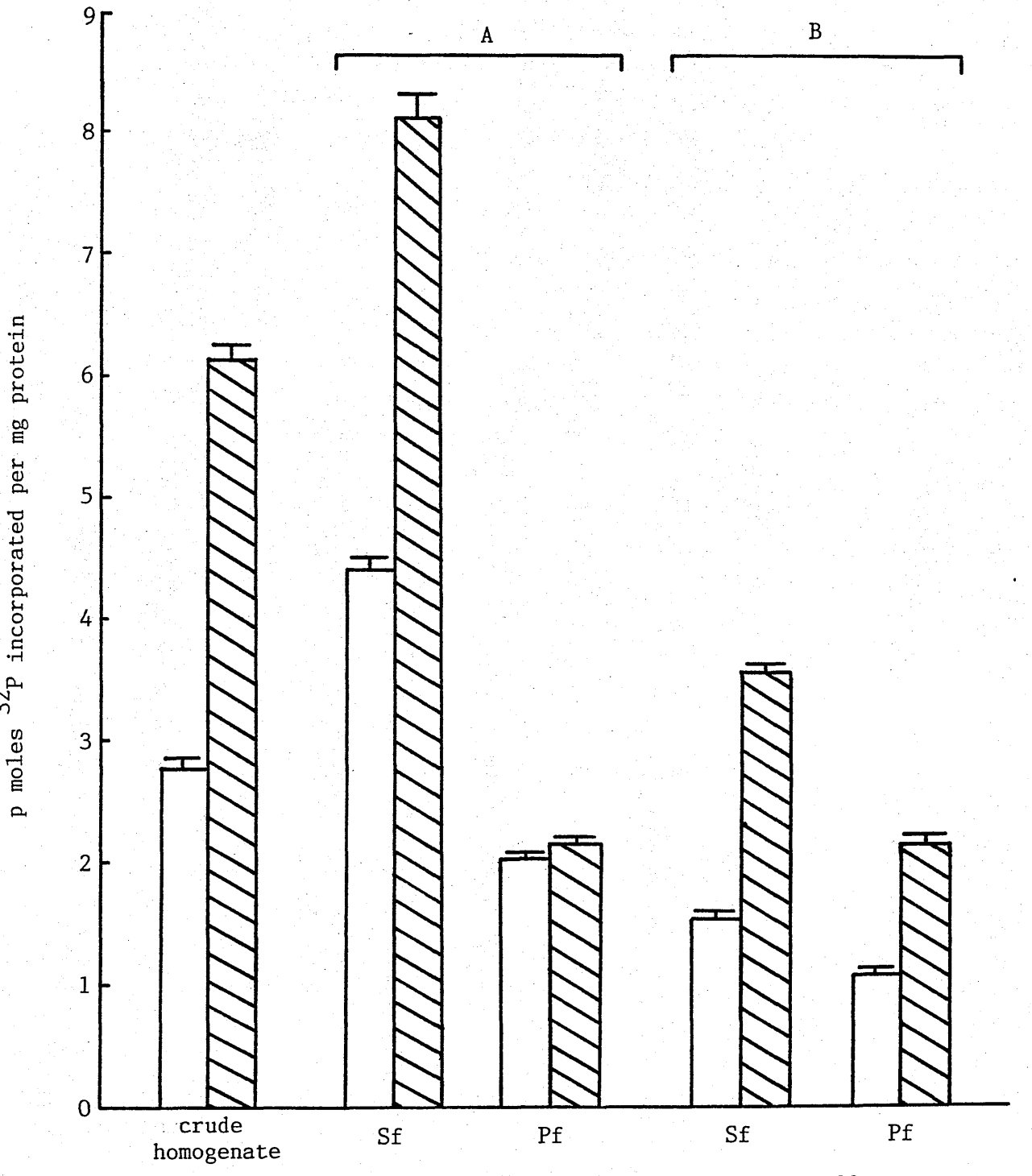


Figure: 32 Electron micrograph of the Pf showing many sheet-like structures resembling broken membranes.

(Magnification x 5,000)

Figure: 33 The Effect of Cyclic AMP on Total Protein Phosphorylation in the Sf and Pf



Aliqouts of the Sf and Pf (Section 2:5:1:1) were incubated with [γ^{32}P] ATP (A) for 1 minute in the absence (□) and presence of 10^{-5}M cyclic AMP (▨) or Sf and Pf were prepared after incubation of the crude homogenate with [γ^{32}P] ATP (B) in the absence (□) and presence of 10^{-5}M cyclic AMP (▨), (Section 2:5:4:1) and the radioactivity present in 'washed' protein pellets was measured. Values are the means ± standard deviations from 3 separate incubations.

This indicates that the Pf contains substrate(s) for cyclic AMP-dependent phosphorylation and that the lack of effect of cyclic AMP was possibly due to the protein kinase being removed during preparation of the Pf. It is possible that the cyclic AMP-dependent protein kinase from Schistocerca gregaria cerebral ganglion is easily solubilized unlike that of rat brain, (Maeno et al, 1971). It is also possible that a soluble factor is necessary for the stimulation of a particulate cyclic AMP-dependent protein kinase.

The effect of exogenous cyclic AMP-dependent protein kinase on the phosphorylation of total protein from the Pf was studied. Aliquots of the Pf were incubated with [γ^{32}P] ATP, (Section 2:5:2) in the presence and absence of cyclic AMP and bovine adrenal cortex cyclic AMP binding protein.

Cyclic AMP did not have a significant effect on total protein phosphorylation in either the Pf or binding protein alone or Pf with either 60 µg/ml or 30 µg/ml binding protein, (Table 13). The radioactivity present in 'washed' protein pellets from incubations with Pf + binding protein was less than that from incubations with Pf alone. A significant increase was observed in the Pf with 15 µg/ml binding protein.

Table: 13 The Effect of Exogenous Protein Kinase on the cyclic AMP-dependent Phosphorylation of Total protein from the Pf

Additions to incubations	Radioactivity recovered in 'washed' protein pellets (cpm) Expt.1 bp = 60µg/ml	Expt.2 bp = 60µg/ml	Expt.3 bp = 30µg/ml	Expt.4 bp = 15µg/ml
Pf only	5,001 ± 125	7,543 ± 221	4,283 ± 121	6,126 ± 134
Pf + cAMP	5,252 ± 152	7,891 ± 202	4,392 ± 93	6,282 ± 97
Pf + bp	4,171 ± 138	7,089 ± 314	4,212 ± 98	6,120 ± 148
Pf + bp + cAMP	4,481 ± 277	7,156 ± 239	4,299 ± 73	6,583 ± 221 *
bp only	1,897 ± 81	2,022 ± 128	882 ± 61	921 ± 104
bp + cAMP	1,833 ± 98	2,056 ± 103	879 ± 68	1,113 ± 73

Pf prepared as described in Section 2:5:1:1 was incubated with [γ^{32}P] ATP in the presence and and absence of bovine adrenal cortex cyclic AMP binding protein (bp) and cyclic AMP (10^{-5}M) as above. The radioactivity bound to total protein was then measured (Section 2:5:2).

* denotes significantly greater than Pf + bp ($P < 0.05$).

Membrane enriched fractions possess a higher adenosinetriphosphatase, (ATPase) activity than other subcellular fractions from rat brain, (Johnson et al, 1971). If the Pf, being a crude membrane fraction, had a higher ATPase activity than either the Sf or crude homogenate, less [γ^{32}P] ATP would be available for protein phosphorylation in incubations with the Pf.

The results from previous experiments indicated that if the level of [γ^{32}P] ATP was decreased, the difference in the level of radioactivity in total protein between control and cyclic AMP also decreased, (Figure 27). These results suggested that if the level of [γ^{32}P] ATP was sufficiently low, a cyclic AMP stimulated increase in the incorporation of radioactivity into total protein would not be detected.

The ATPase activity of the Pf, Sf and crude homogenate was measured by incubating with ATP and measuring the formation of inorganic phosphate, (Section 2:6).

The highest relative specific ATPase activity (which was expressed as a ratio of that of the crude homogenate) resided in the Pf and the lowest in the Sf. The values obtained for the Pf and Sf were 1.72 ± 0.31 and 0.91 ± 0.22. The relative total ATPase activity (also expressed as a ratio of that of the crude homogenate but corrected for the actual amounts of protein in the protein phosphorylation assays) of the Pf (1.34 ± 0.21) was slightly more than double that of the Sf, (0.61 ± 0.18). Experiments were then carried out in which

the Pf was incubated with twice the normal level of [γ^{32}P] ATP and unlabelled ATP. Under these conditions cyclic AMP significantly increased (probability value of < 5%) the incorporation of radioactivity into total protein from 7.28 ± 0.08 p moles ^{32}P incorporated per mg protein in control preparations to 7.65 ± 0.21 in cyclic AMP preparations, (values are the means ± standard deviations of 3 separate incubations).

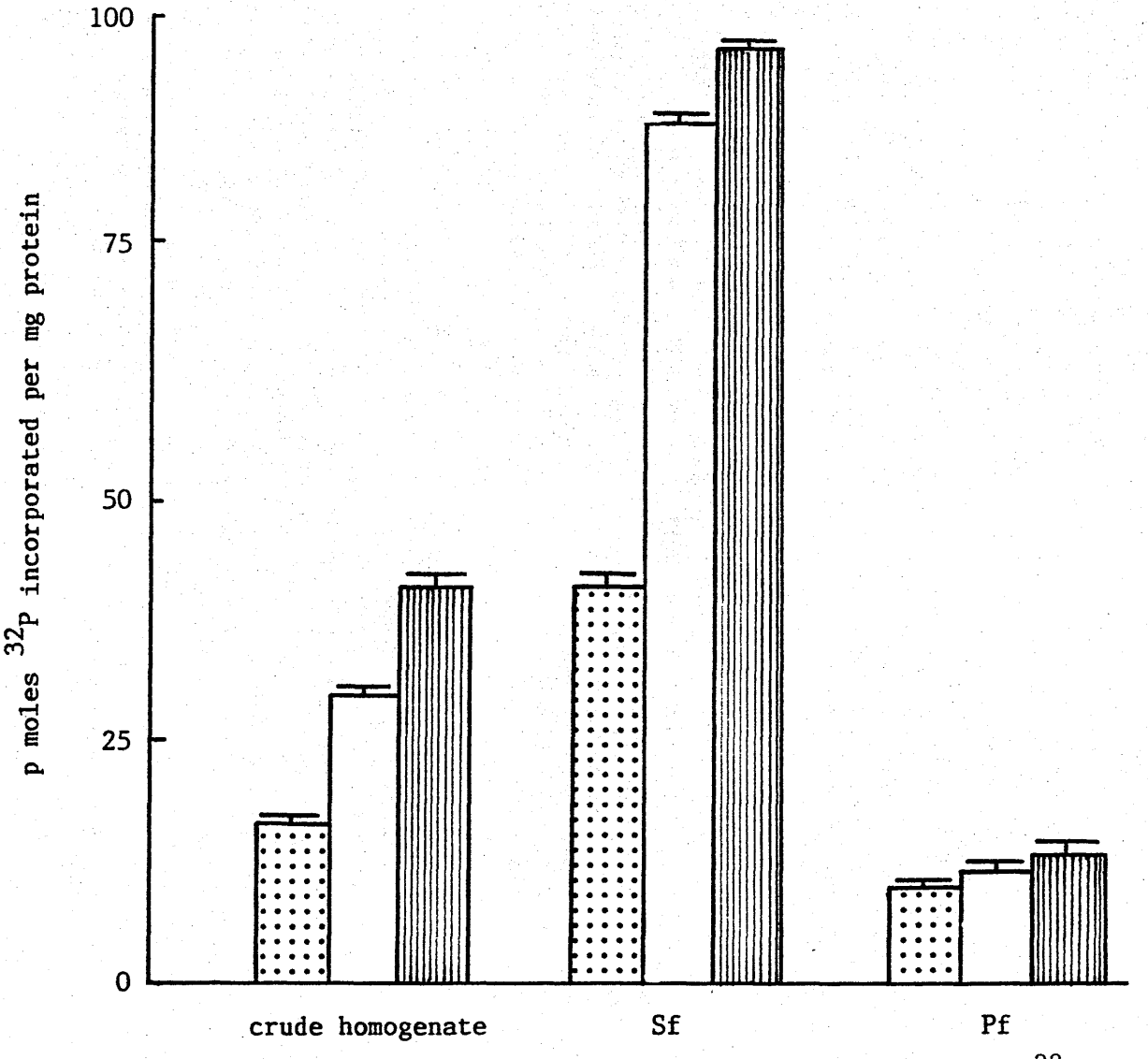
It appears that although protein substrate(s) exist for cyclic AMP-dependent protein kinase in the Pf, (Figure 33) this fraction has a very low intrinsic cyclic AMP-dependent protein kinase activity in comparison to the crude homogenate or Sf.

3:2:8:1b <u>The Effect of Calcium</u>

The effect of Ca^{2+} on the incorporation of radioactivity into total protein from Sf and Pf was studied.

Addition of $CaCl_2$ (250 μM) to the incubation medium increased the incorporation of radioactivity into total protein from crude homogenate and the Sf but not the Pf, (Figure 34). Addition of EGTA (700 μM) to the incubation medium reduced the incorporation of radioactivity into total protein from all 3 fractions in the absence of added calcium. This suggested that basal phosphorylation of total protein in the absence of EGTA is stimulated by endogenous calcium.

Figure: 34 The Effect of Calcium on Total Protein Phosphorylation in the Sf and Pf

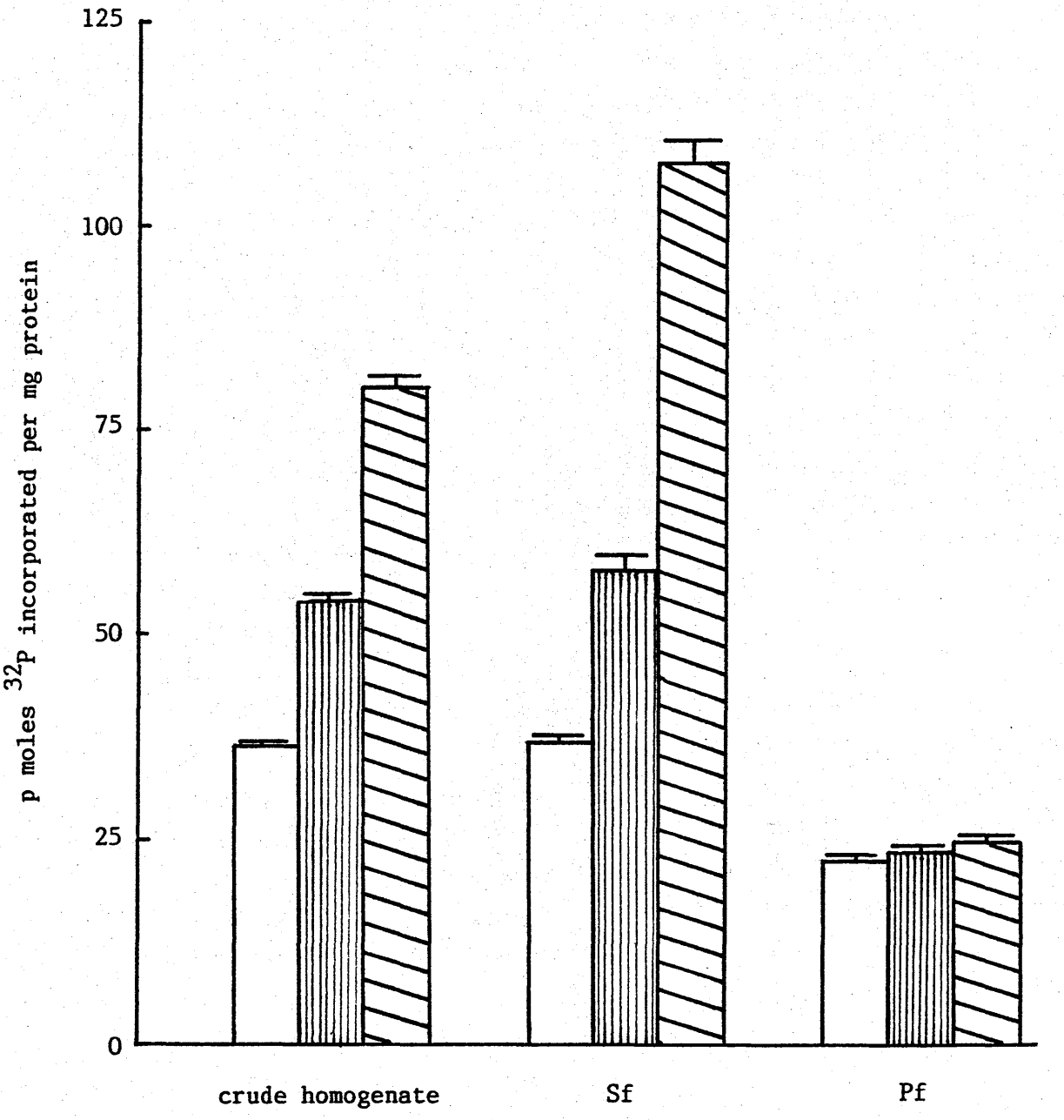


Aliqouts of tissue fractions (Section 2:5:1:1) were incubated with [γ^{32}P] ATP for 1 minute in the presence of 0.7mM EGTA only (▦), 0.25mM $CaCl_2$ only (▥) with no EGTA and buffer only without EGTA (□). The radioactivity present in 'washed' protein pellets was then measured (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

3:2:8:1c Direct Comparison of the Effect of Calcium and Cyclic AMP

Crude homogenate, Sf and Pf were incubated with [γ^{32}P] ATP and $CaCl_2$ (1,000 μM) or cyclic AMP (10 μM) in the presence of EGTA (700 μM). Under these conditions the stimulation of protein phosphorylation by cyclic AMP in the Sf and crude homogenate was greater than that of calcium, (Figure 35). Both cyclic AMP and calcium stimulated a small increase in the phosphorylation of total protein from the Pf.

Figure: 35 Direct Comparison of the Effect of Cyclic AMP and Calcium on Total Protein Phosphorylation in the Sf and Pf



Aliquots of tissue fractions (Section 2:5:1:1) were incubated for 1 minute with [γ^{32}P] ATP in the absence (▭) or presence of either 1mM $CaCl_2$ (▥) or 10^{-5}M cyclic AMP (▨) and the radioactivity present in 'washed' protein pellets was measured, (Section 2:5:2). EGTA (0.7 mM) was present in all incubations. Values are the means ± standard deviations from 3 separate incubations.

3:2:8:2 10,000 g Fractions prepared in 0.25 M Sucrose

Cerebral ganglia were homogenized in 0.25 M sucrose (Section 2:5:1:4) and centrifuged at 10,000 g. Samples of the 10,000 g pellet were examined by electron microscopy. Electron micrographs (Figure 36) revealed that the pellet contained synaptosome structures similar to those reported by Breer, (1981).

Cyclic AMP (10^{-5} M) increased the incorporation of radioactivity into total protein from crude homogenate, 10,000 g supernatants and 10,000 g pellets incubated with [γ^{32}P] ATP, (Figure 37). The highest incorporation of radioactivity into total protein occured in the 10,000 g supernatant in both cyclic AMP and control preparations.

The time course of protein phosphorylation in the 10,000 g supernatant and pellet was studied between 30 seconds and 2 minutes. Aliquots of 10,000 g supernatant and pellet were incubated with [γ^{32}P] ATP in the presence and absence of cyclic AMP (10^{-5} M).

Incorporation of radioactivity into total protein from 10,000 g supernatant increased in a curvilinear manner in both control and cyclic AMP preparations, (Figure 38). The rate of incorporation into cyclic AMP preparations was higher than in control preparations.

The radioactivity incorporated into total protein from the 10,000 g pellet decreased with time in both control and cyclic AMP preparations. The rate of decrease was slightly greater

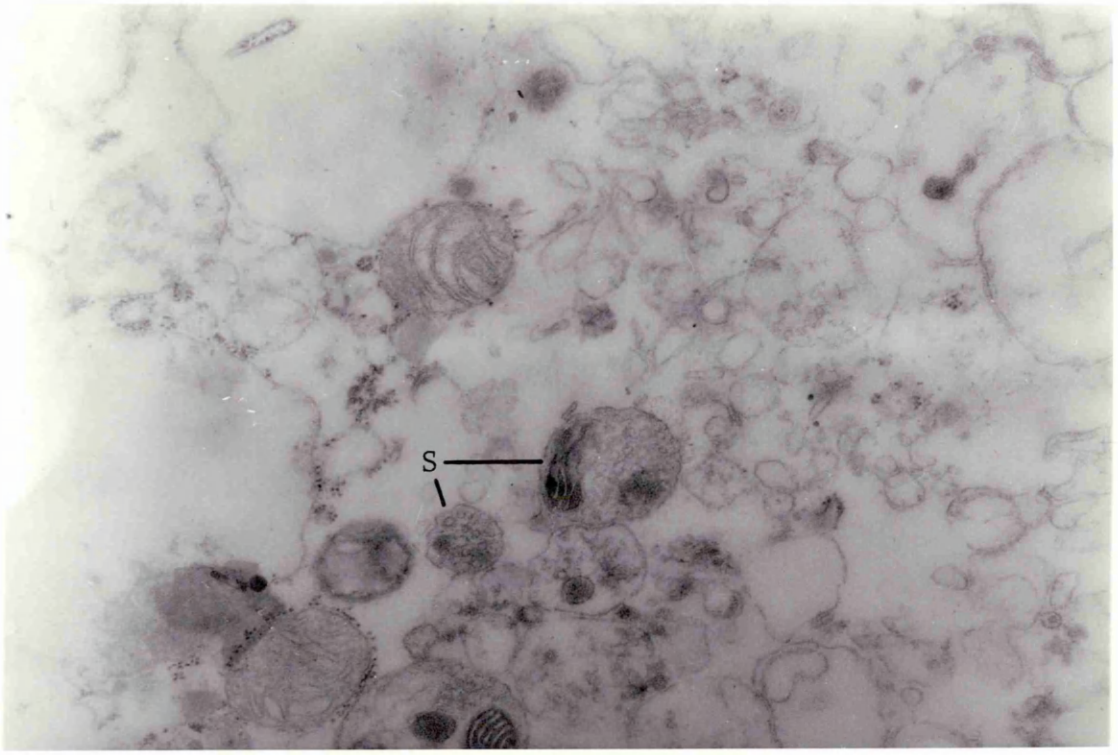
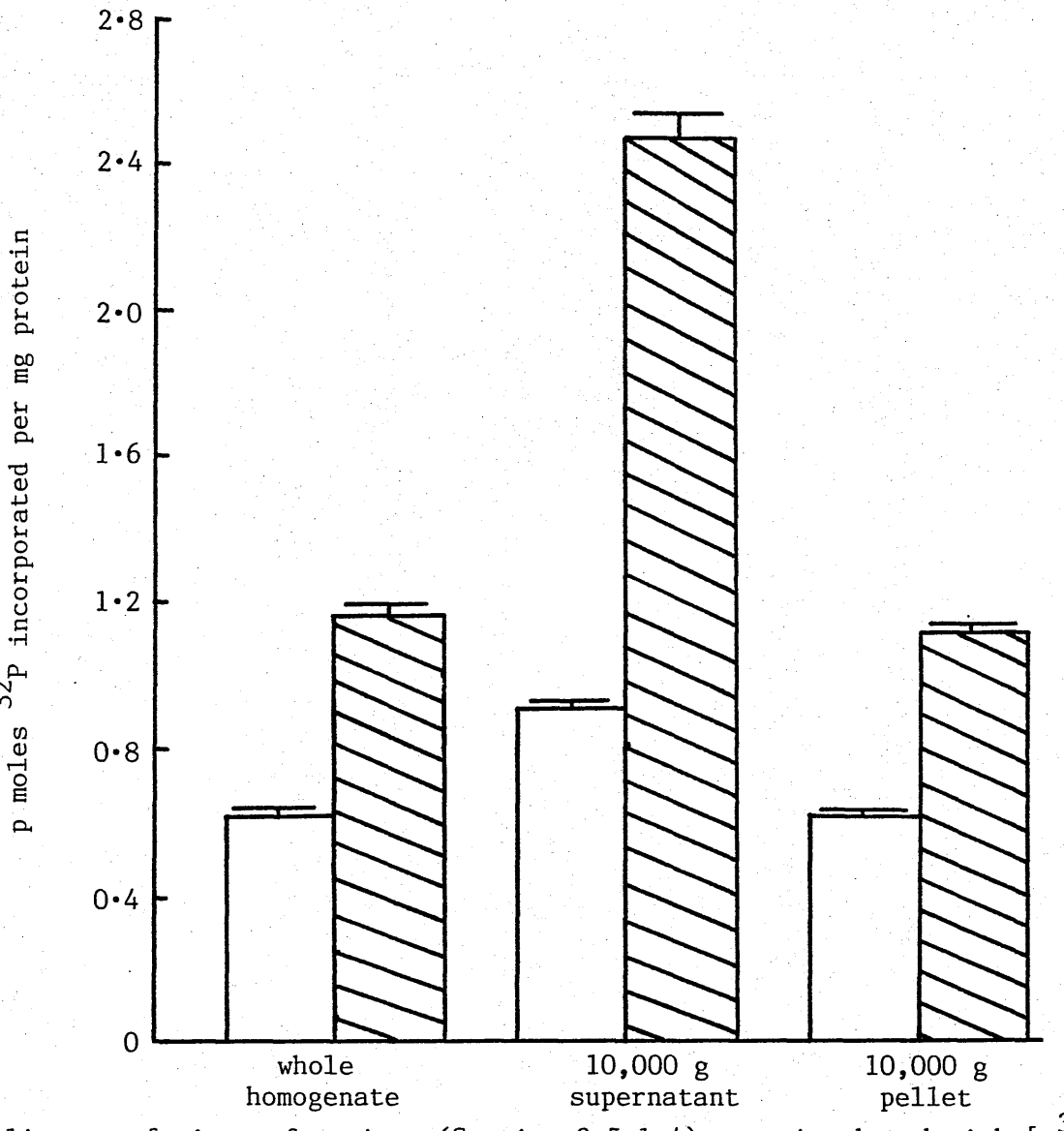


Figure: 36 Electron micrograph of the 10,000 g pellet prepared in 0.25 M sucrose (Section 2:5:1:4) showing synaptosome structures (S) as reported by Breer, (1981) and containing vesicles and mitochondria.

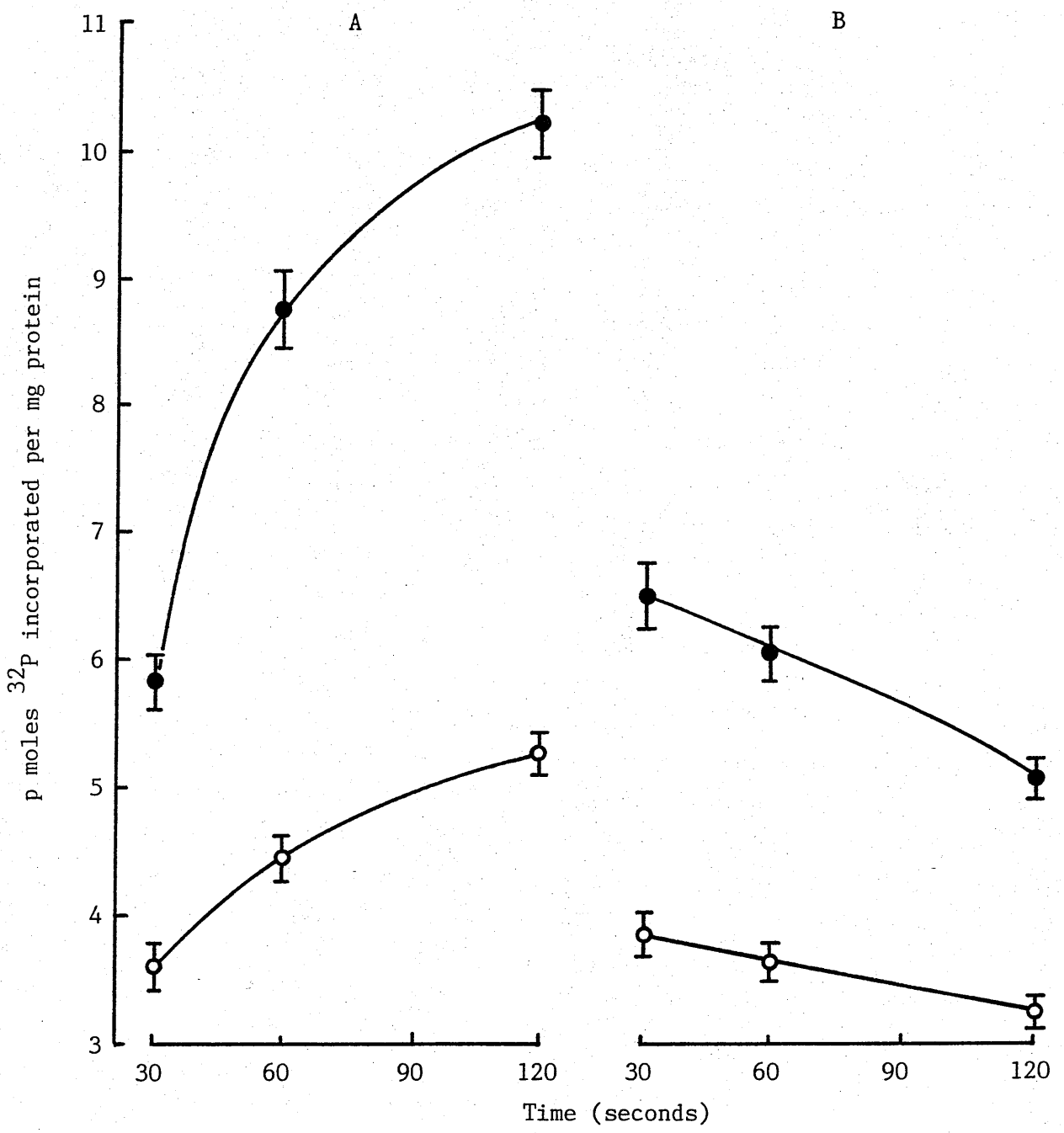
(Magnification x 20,000)

Figure: 37 The Effect of Cyclic AMP on Total Protein Phosphorylation in 10,000 g Fractions prepared in 0.25 M Sucrose



Aliqouts of tissue fractions (Section 2:5:1:4) were incubated with [γ^{32}P] ATP for 1 minute in the absence (□) or presence of 10^{-5}M cyclic AMP (▨) and the radioactivity present in 'washed' protein pellets was measured (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

Figure: 38 Time Course of Total Protein Phosphorylation in 10,000 g Fractions prepared in 0.25 M Sucrose



Aliquots of 10,000 g supernatants (A) and 10,000 g pellets (B) prepared as described in Section 2:5:1:4 were incubated with [γ^{32}P] ATP for various times in the absence (O) and presence of 10^{-5}M cyclic AMP (●) and the radioactivity present in 'washed' protein pellets was measured, (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

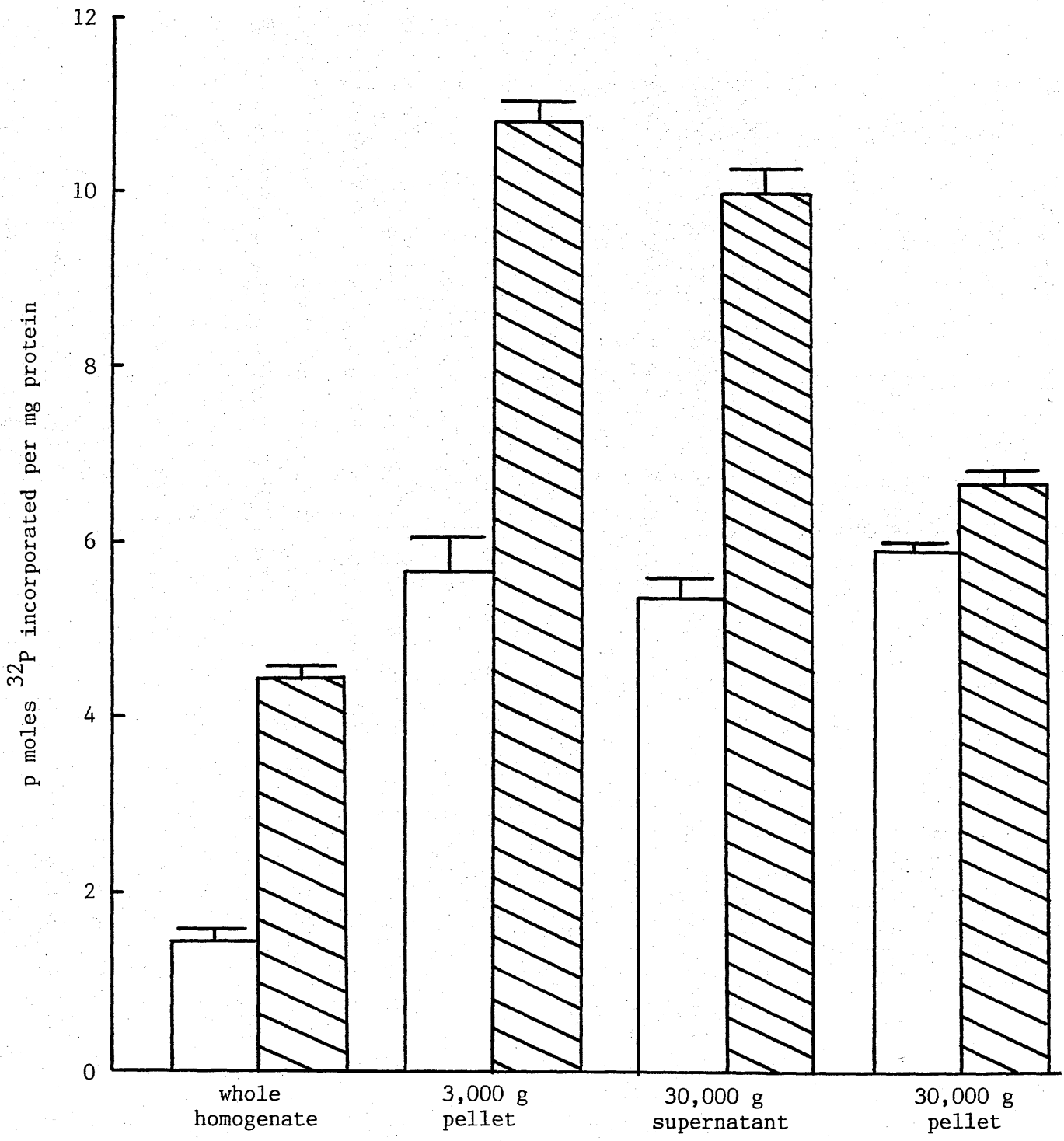
in cyclic AMP preparations.

3:2:8:3 Crude Synaptosomes

Protein phosphorylation was measured in a crude synaptosomal fraction prepared as described in section 2:5:1:3 by a method similar to that of Kelly, (1981). Protein phosphorylation was also measured in other fractions (crude homogenate, 3,000 g pellet and 30,000 g supernatant) obtained in the preparation of crude synaptosomes (30,000 g pellet). Fractions were incubated with [γ^{32}P] ATP in the presence and absence of cyclic AMP (10^{-5} M) and the radioactivity present in 'washed' protein pellets was measured.

Cyclic AMP increased the incorporation of radioactivity into total protein from the crude homogenate, 3,000 g pellet and 30,000 g supernatant. A much smaller but significant increase was observed in cyclic AMP preparations from the crude synaptosome fraction, (Figure 39).

Figure: 39 The Effect of Cyclic AMP on Total Protein Phosphorylation in Crude Synaptosomes



Aliquots of tissue fractions obtained in the preparation of crude synaptosomes (30,000 g pellet) as described in Section 2:5:1:3 were incubated for 1 minute with [γ^{32}P] ATP in the absence (□) and presence of 10^{-5}M cyclic AMP (▨). The radioactivity present in 'washed' protein pellets was then measured (Section 2:5:2). Values are the means ± standard deviations from 3 separate incubations.

3:2:9 Phosphorylation of Specific Proteins in Homogenized Tissue

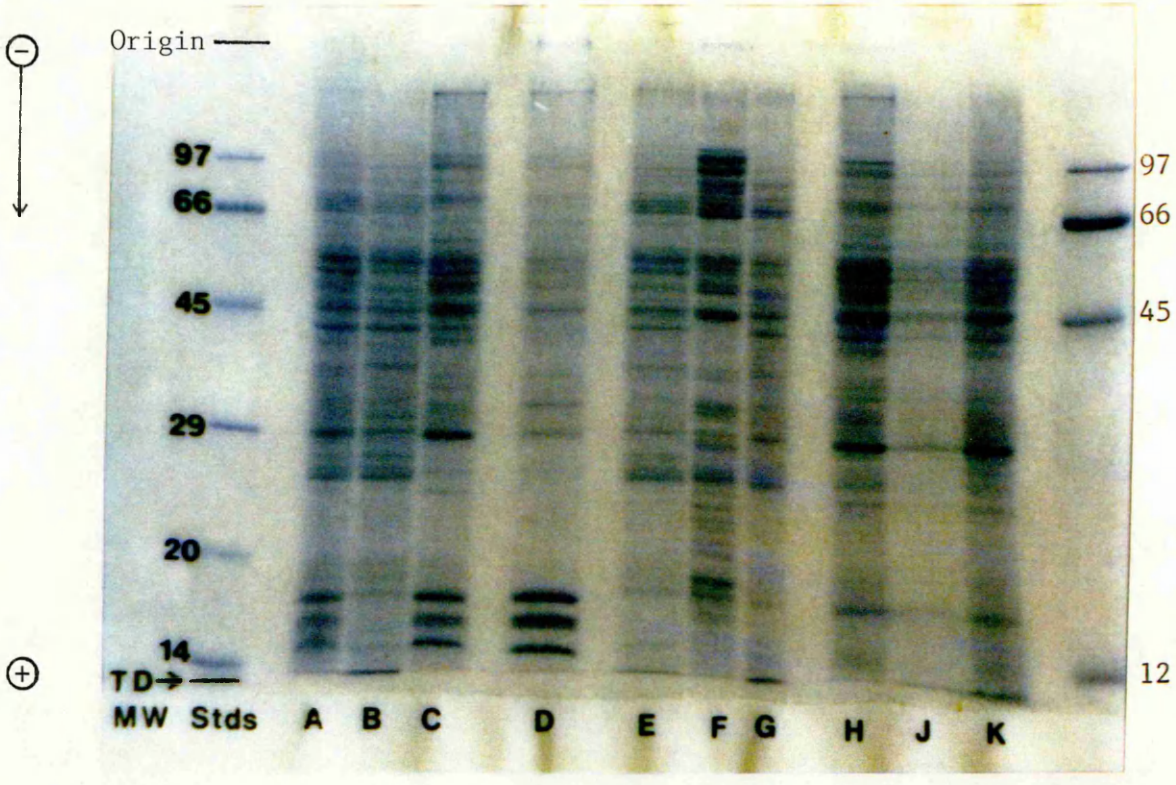
The phosphorylation of specific proteins was studied by incubating tissue fractions with [γ^{32}P] ATP followed by the addition of an SDS/solubilizing buffer. The solubilized proteins were then separated by polyacrylamide/SDS slab-gel electrophoresis, (Section 2:5:3), and the distribution of radioactivity measured by autoradiography. Figure 40 shows the Coomassie blue-stained protein profiles of the tissue fractions studied.

3:2:9:1 The Effect of Octopamine, Cyclic AMP and GppNHp

Homogenates of cerebral ganglia were pre-incubated for 5 minutes with unlabelled ATP (100 μM) and the appropriate test substance prior to the addition of [γ^{32}P] ATP. Incubations were then continued for a further 1 minute.

Octopamine (10^{-4} M) in the presence of GTP (10^{-4} M) increased the incorporation of radioactivity into 3 protein bands (M_r: 51,000; 39,000 and 21,000). The incorporation of ^{32}P into these 3 proteins was greater than with octopamine alone, (Figure 41). GppNHp (10^{-4} M) alone also increased the incorporation of ^{32}P into these 3 proteins. Cyclic AMP (10^{-6} M) stimulated an increase in phosphorylation of at least 8 proteins ranging from M_r 21,000 to 93,000, (see also Figure 44).

Figure: 40 Protein Profiles of Tissue Fractions in Polyacrylamide/SDS Gels Stained with Coomassie Brilliant Blue



Tissue fractions prepared as described in the relevant sections[1] were dissolved in electrophoresis solubilizing buffer and aliquots (adjusted to contain 35 µg protein) were electrophoresed on 10% polyacrylamide/SDS gels, (Sections 2:3:4:2/3).

Key to figure:

A) Crude homogenate, B) Sf, C) Pf, D) 700 g pellet, E) 25,000 g supernatant, F) 150,000 g supernatant, G) 150,000 g pellet, H) 25,000 g pellet, J) Ficoll pellicle (synaptosomes), K) Ficoll pellet (mitochondria).

Molecular weight standards were (x 10^{-3}) phosphorylase b (97), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (29), soybean trypsin inhibitor (20), α-lactalbumin (14) and cytochrome c (12).

TD denotes tracking dye.

[1] tracks A, B and C (Section 2:5:1:1), tracks D-K (Section 2:5:1:2).

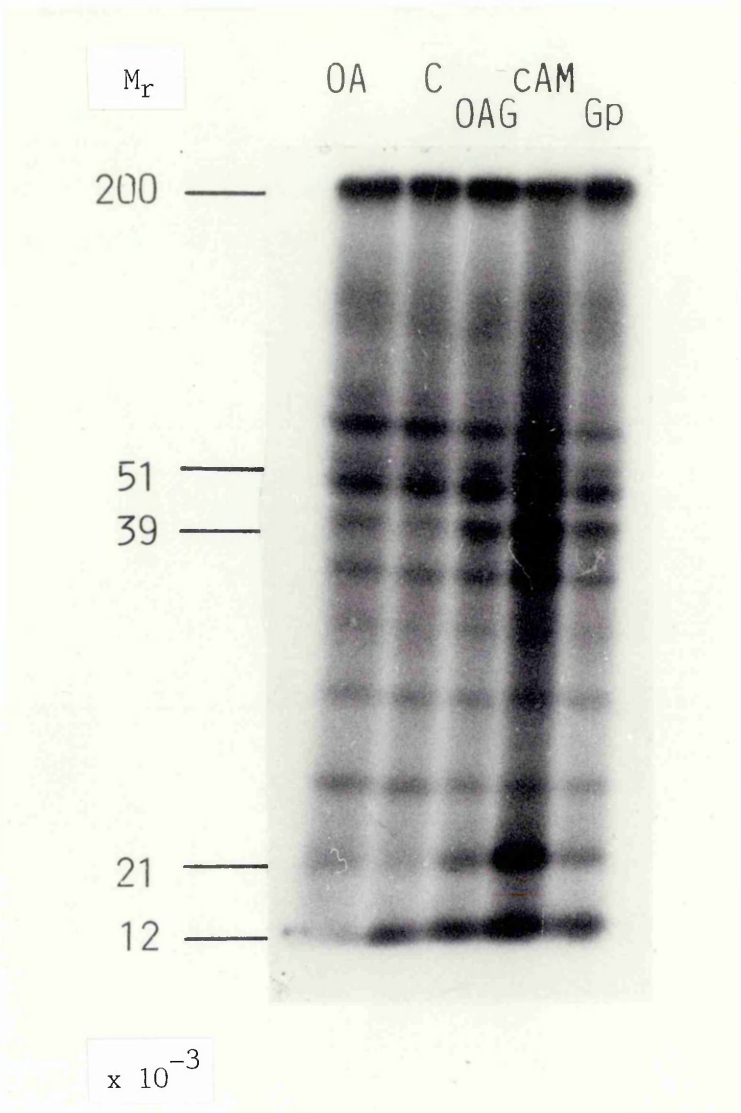


Figure: 41 The effect of octopamine, GppNHp and cyclic AMP on specific protein phosphorylation in homogenates of cerebral ganglia. Homogenates were pre-incubated for 5 minutes with unlabelled ATP (100 μM) in the absence or presence of either 10^{-4} M octopamine (OA), 10^{-4} M octopamine + 10^{-4} M GTP (OAG), or 10^{-4} M GppNHp (Gp) after which [γ^{32}P] ATP was added for 1 minute, (Section 2:5:4:2). Incubations with 10^{-6} M cyclic AMP (cAM) were carried out as described in Section 2:5:4:2. Homogenates were solubilized by the addition of an SDS buffer followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:3:4:1/2). Figure shows autoradiograph of gel.

3:2:9:2 The Effect of Chloroform/methanol and Pronase treatment

Tissue homogenates were incubated with [γ^{32}P] ATP in the presence or absence of cyclic AMP (10^{-5} M) as described in section 2:5:3. The TCA precipitated pellets were then washed with chloroform/methanol and solubilized for electrophoresis, (Section 2:5:5).

Treatment of the TCA pellets with chloroform/methanol did not alter the pattern of radioactivity in polyacrylamide gels when compared to pellets which had not been treated, (Figure 42). The chloroform/methanol washes were collected and solubilizing buffer added in the same proportion as described in section 2:5:5. The radioactive pattern in gels from this was similar to that of the chloroform/methanol treated and untreated pellets, although the radioactivity in every band (except that at M_r 21,000) was much less. This suggested that a small proportion of the protein from pellets was removed with the chloroform/methanol, (see also Section 3:2:2) although these proteins were the same and not selectively removed with the possible exception of the band at M_r 21,000. This band persisted in the chloroform/methanol washes, chloroform/methanol treated TCA precipitates and the untreated TCA precipitates, (Figure 42) and may be a proteolipid. Much of the radioactivity migrating well ahead of the tracking-dye (which co-migrated with the 12,000 molecular weight marker, (cytochrome C), in untreated pellets, was not present in chloroform/methanol treated pellets. This radioactivity was, however, present in the chloroform/methanol washes. It appears that this radioactivity was selectively removed by the chloroform/methanol washes and may be due to phospholipids. It does not

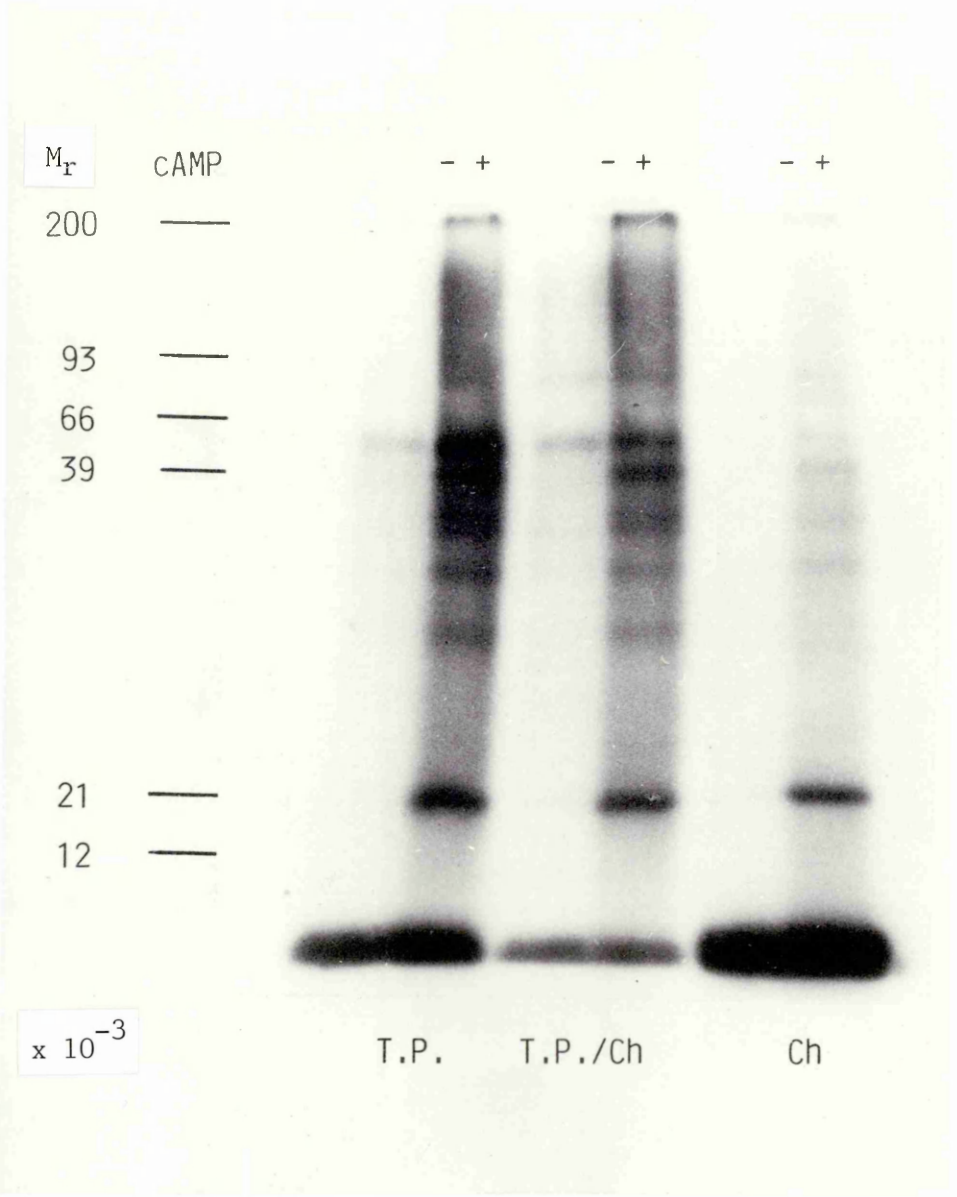


Figure: 42 Chloroform/methanol treatment of TCA insoluble material. Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the absence (-) or presence of 10^{-5}M cyclic AMP (+) for 1 minute after which TCA was added, (Section 2:5:2). The TCA pellet (T.P.) was either dissolved directly in 50 µl solubilizing buffer (Section 2:3:4:1) or washed twice with 50 µl acidified chloroform/methanol as in Section 2:3:2 and the residual pellet dissolved in 50 µl solubilizing buffer (T.P./Ch). Solubilizing buffer (50 µl) was also added to the pooled chloroform/methanol washes (Ch) and after thorough mixing the chloroform/methanol was evaporated in a boiling water bath. Aliquots (25 µl) of the solubilized material were electrophoresed on 10% polyacrylamide/SDS gels, (Section 2:3:4). Figure shows autoradiograph of gel.

appear that any of the radioactive bands separated by the gels is due to phospholipids.

TCA precipitated pellets were resuspended in phosphate buffer and incubated for 24 hours in the presence and absence of pronase, (Section 2:5:5) prior to polyacrylamide gel electrophoresis. The radioactive bands obtained on gels from control and cyclic AMP preparations, (Figure 43) were identical to those obtained from TCA precipitated pellets, (Figure 42) which had not been incubated for 24 hours in phosphate buffer.

No radioactivity bands were detected in pronase treated pellets from either control or cyclic AMP preparations. Small amounts of radioactivity migrating ahead of the tracking-dye were, however, detected. These results indicate that all of the radioactivity present in gels behind the tracking-dye was associated with protein.

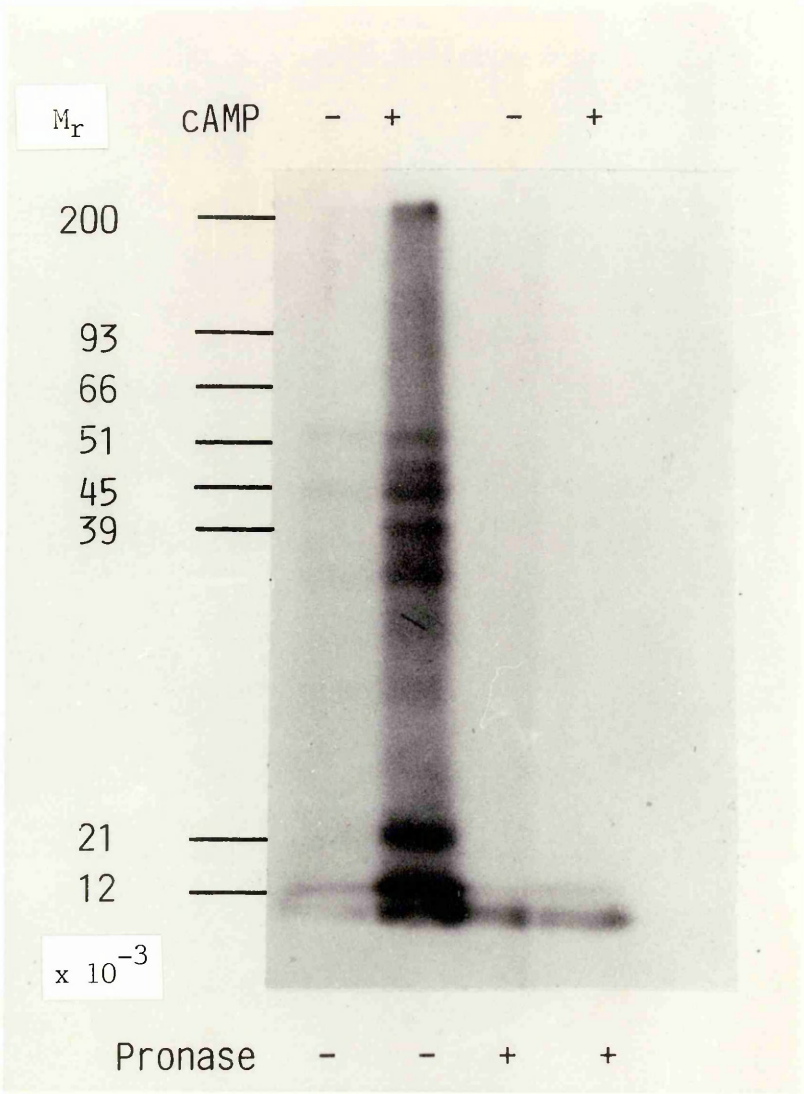


Figure: 43 Pronase treatment of TCA insoluble material. Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the absence (-) or presence of 10^{-5} M cyclic AMP (+) for 1 minute after which TCA was added, (Section 2:5:2). The TCA insoluble material was then resuspended in phosphate buffer and incubated in the absence (-) or presence of pronase (+). SDS solubilizing buffer was added to incubations after 24 hours followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:5). Figure shows autoradiograph of gel.

3:2;9:3 Time Course of the Effect of Cyclic AMP on the Phosphorylation of Specific Proteins

Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the presence and absence of cyclic AMP (10^{-5} M) for various times up to 30 minutes. No radioactive bands were detected in zero-time incubations in which solubilizing buffer and homogenate were added before [γ^{32}P] ATP. Cyclic AMP stimulated the incorporation of ^{32}P into many protein bands after 10 seconds, (Figure 44). The amount of radioactivity present in 8 protein bands with apparent molecular weights between 21,000 and 93,000 which were affected by cyclic AMP, (Figures 45A-H) was measured by cutting out the bands from the gel and measuring the amount of radioactivity by scintillation counting, (Section 2:3:4:3). All of the proteins achieved a maximum incorporation in the presence of cyclic AMP between one and 5 minutes. The highest cyclic AMP-dependent incorporation of ^{32}P occured in the M_r 39,000 and 45,000 proteins. It also appeared that cyclic AMP stimulated an increased incorporation of ^{32}P into a band which migrated with,or at times ahead of the tracking dye ($M_r \leq 12,300$).

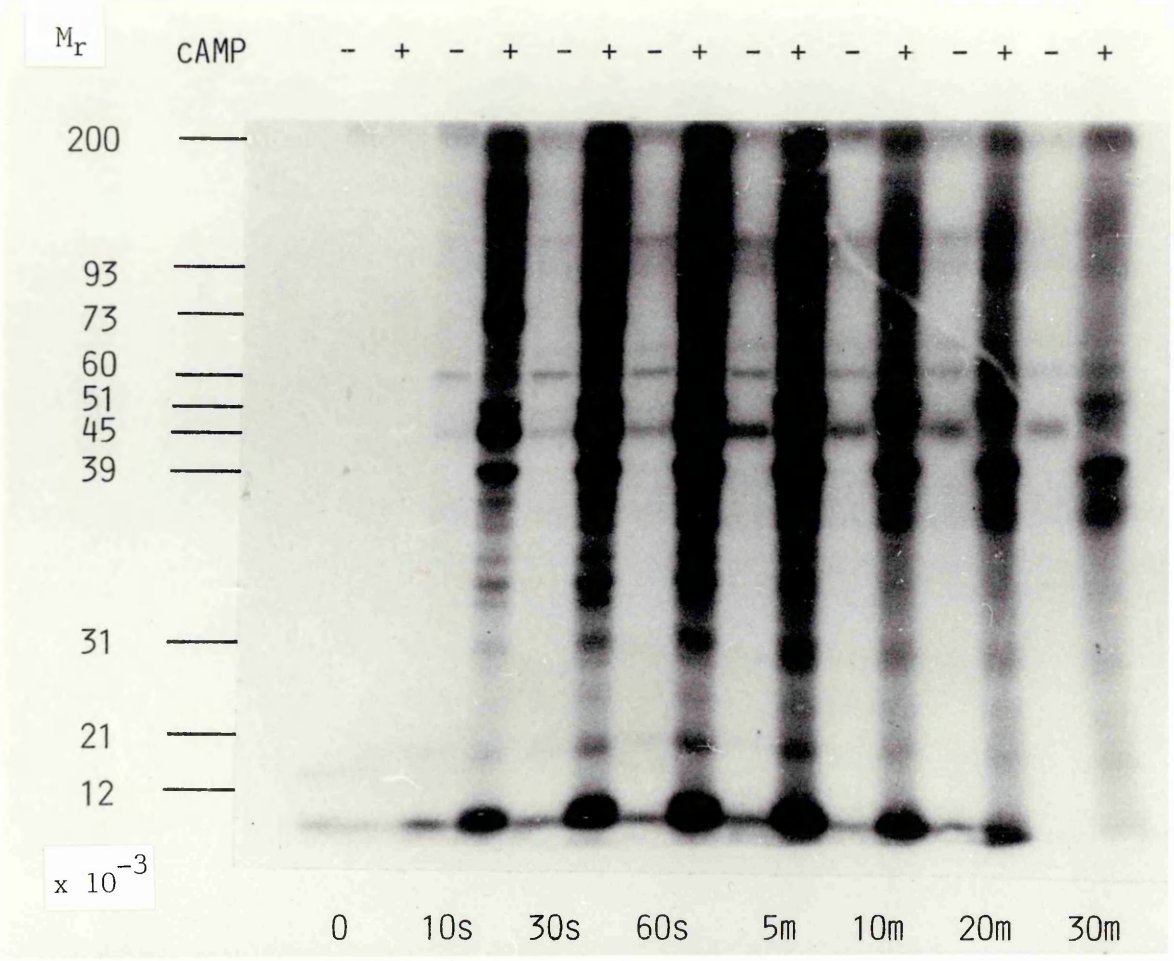
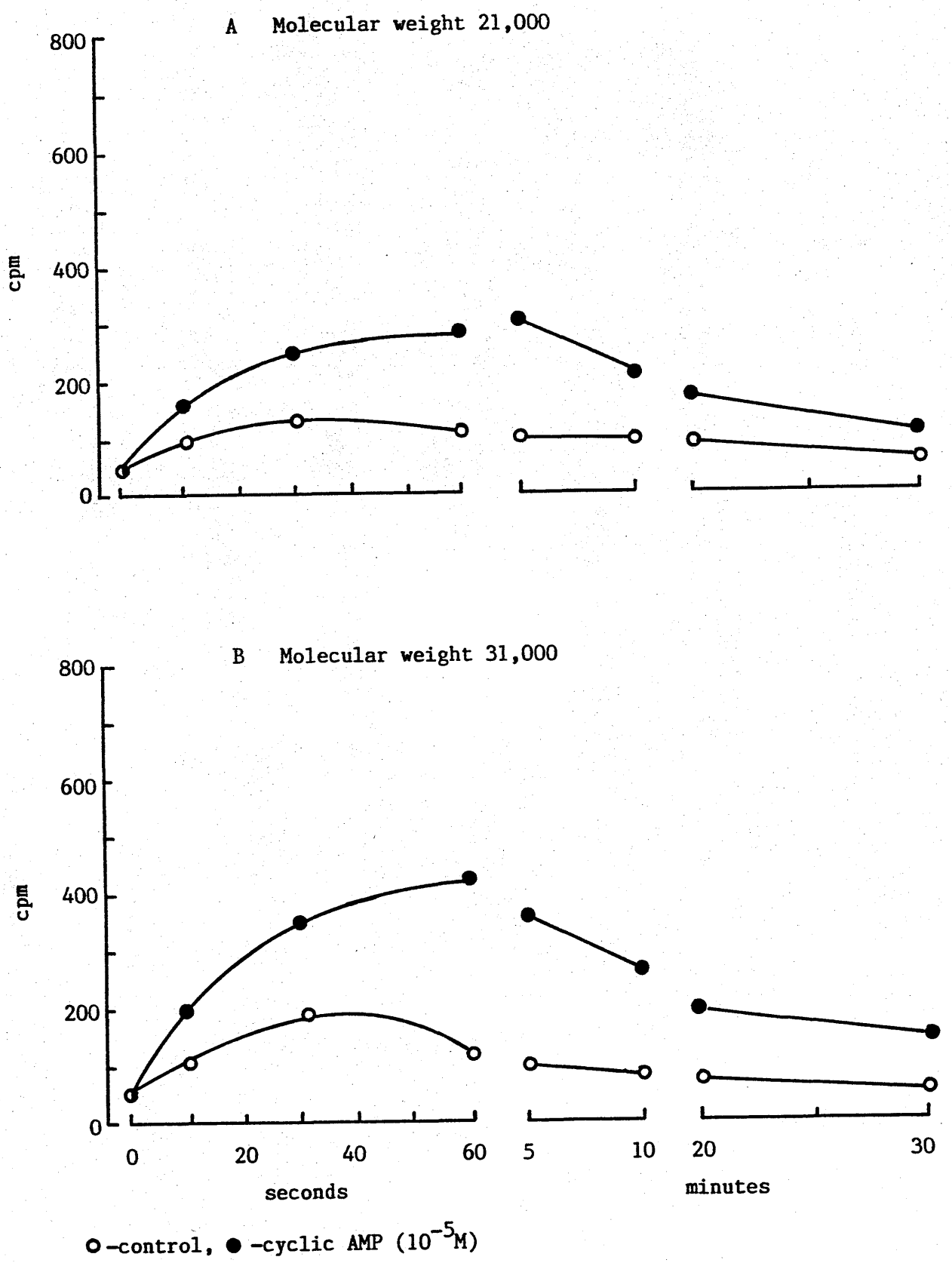


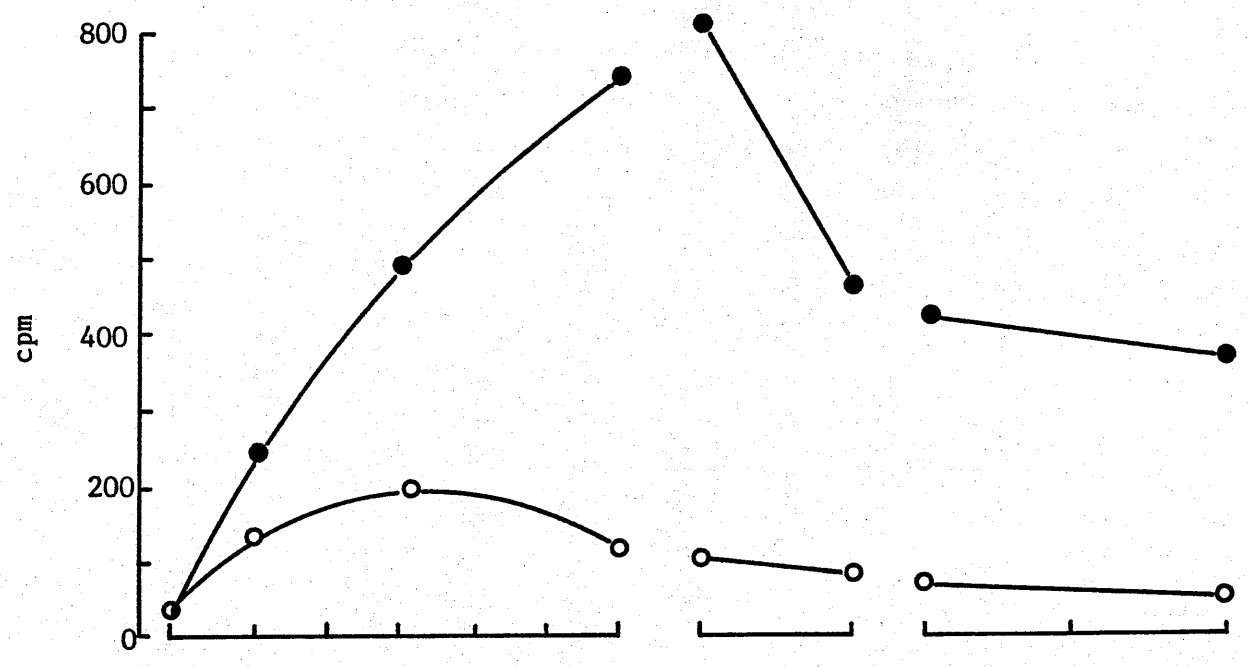
Figure: 44 Time course of specific protein phosphorylation in homogenates. Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the absence (-) or presence of 10^{-5} M cyclic AMP (+) for various times followed by electrophoresis on 10% polyacrylamide gels, (Section 2:5:3). Figure shows autoradiograph of gel.

s denotes seconds and m minutes

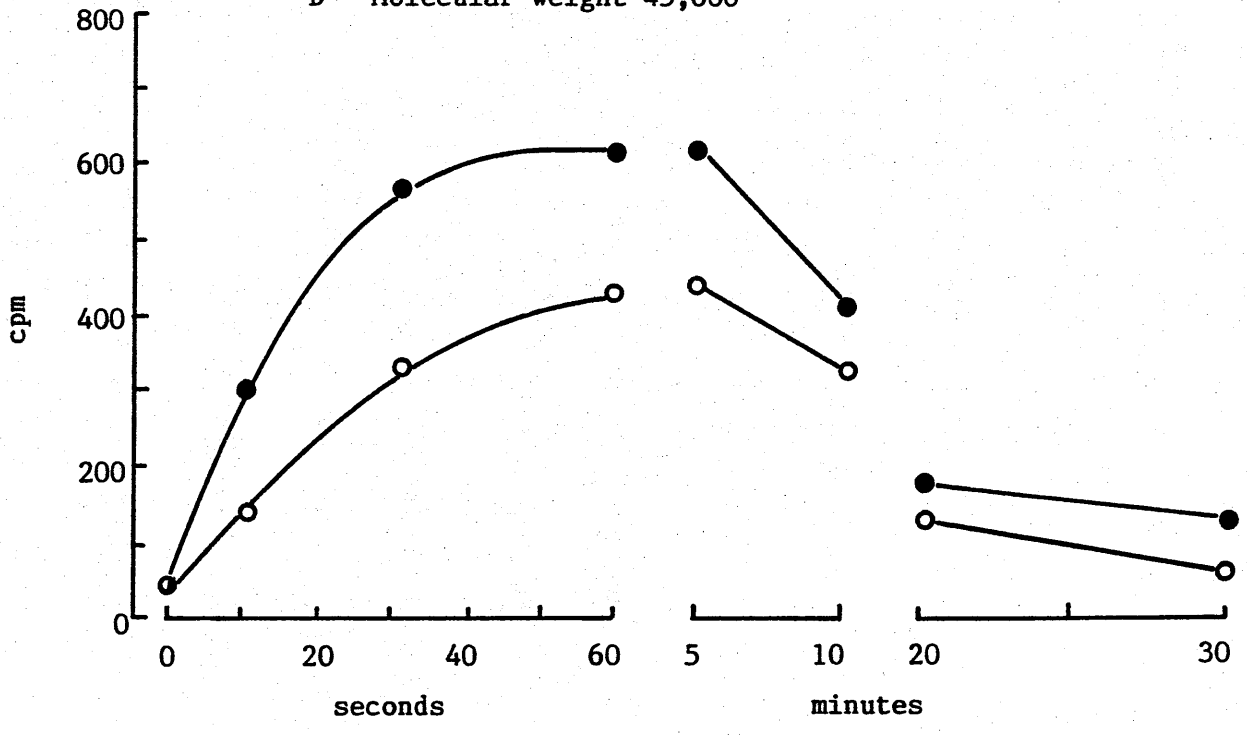
Figures: 45 A-H Quantitative analysis of the time course of specific protein phosphorylation in homogenates. Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the absence (O) and presence of cyclic AMP (●) followed by electrophoresis on 10% polyacrylamide/SDS gels as for Figure 44. Portions of the gel corresponding to the molecular weights indicated in the graphs were cut out and the radioactivity present was measured by liquid scintillation counting (Section 2:3:4:3/4).


A Molecular weight 21,000
800
600
400
200
0
cpm
B Molecular weight 31,000
800
600
400
200
0
cpm
0
20
40
60
5
10
20
30
seconds
minutes
○-control, ●-cyclic AMP (10^{-5}M)

C Molecular weight 39,000

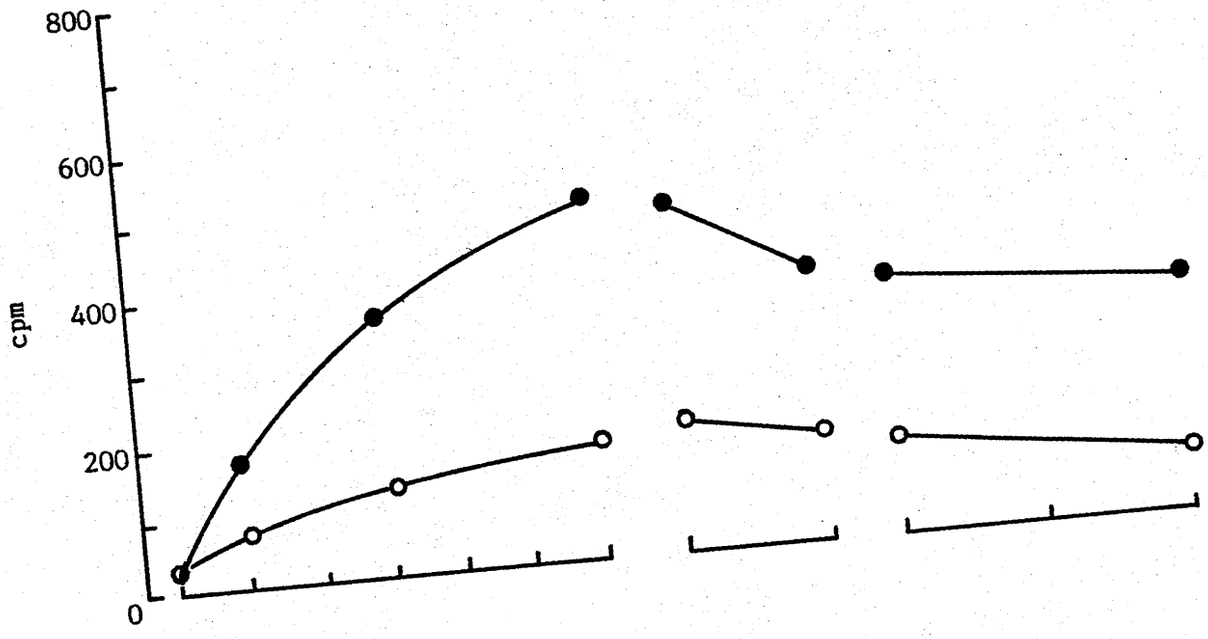


D Molecular weight 45,000

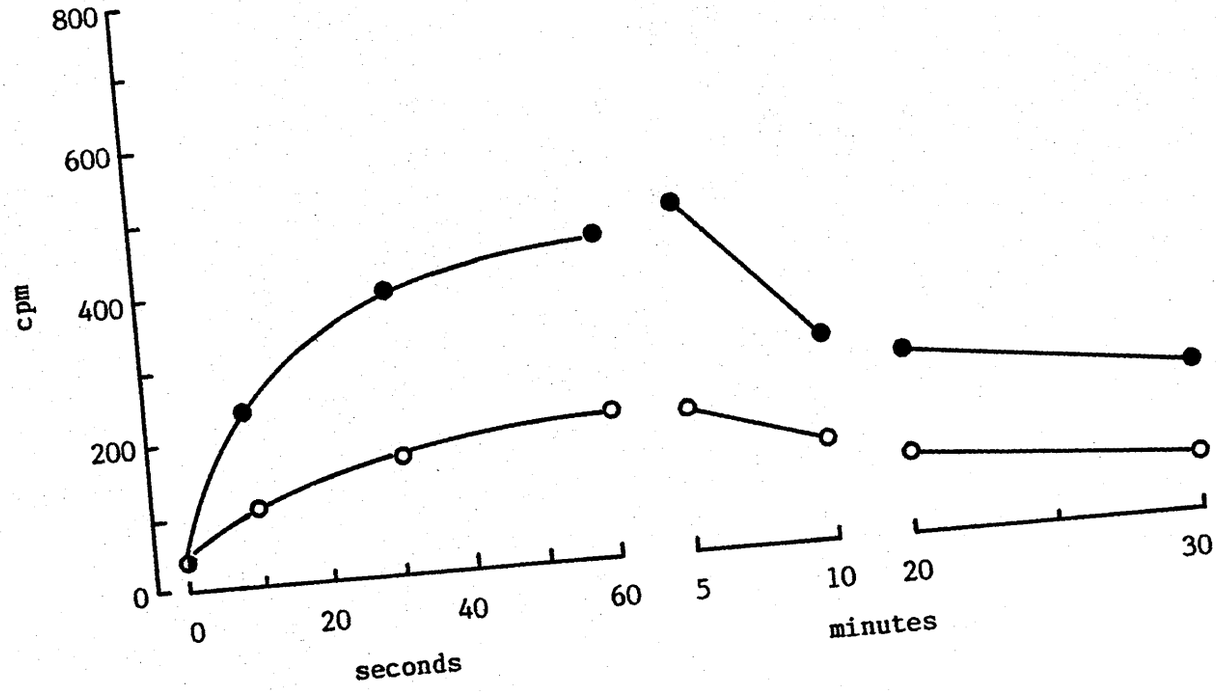


○-control, ●-cyclic AMP (10^{-5}M)

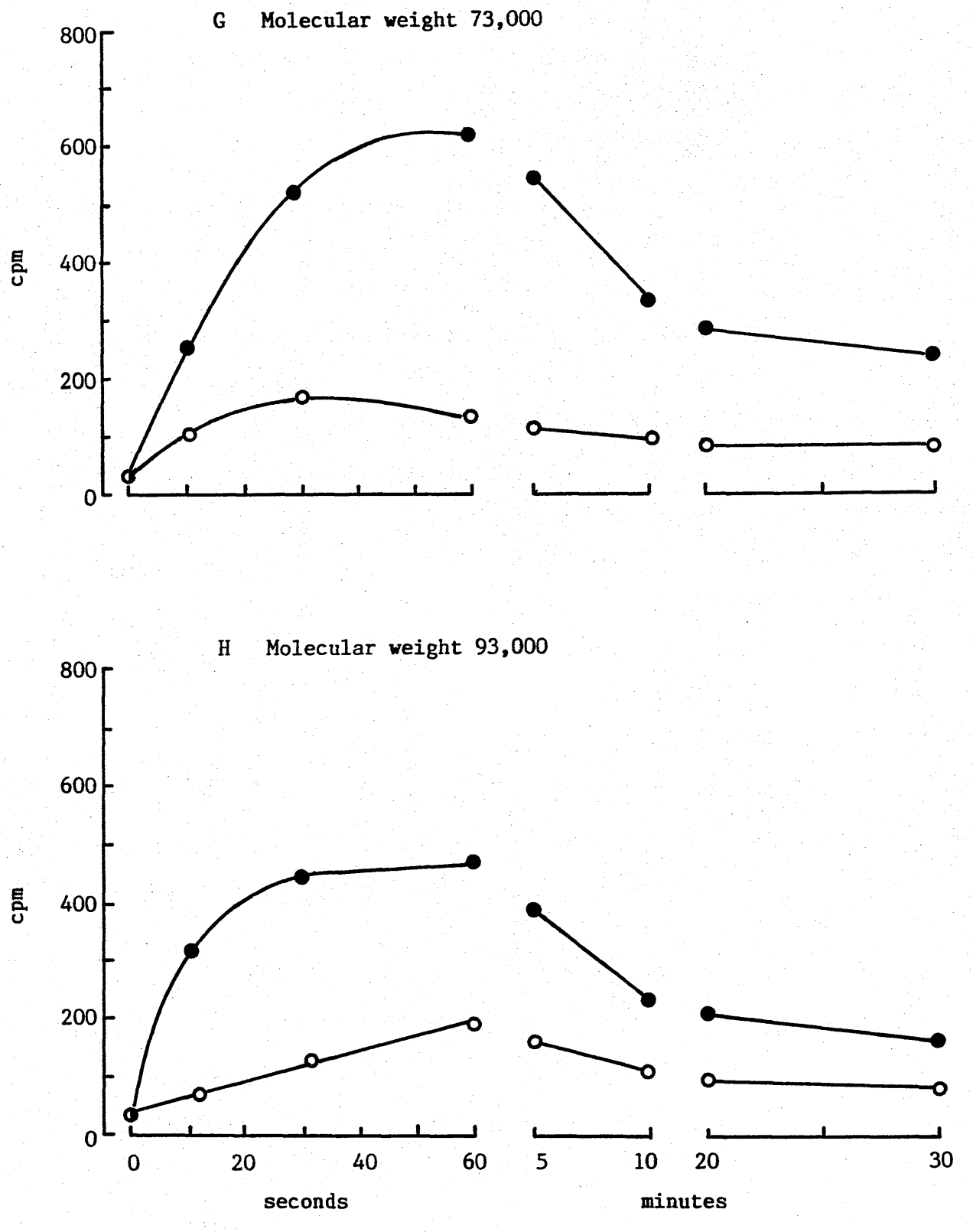
E Molecular weight 51,000



F Molecular weight 60,000



○-control, ●-cyclic AMP (10^{-5}M)


G Molecular weight 73,000
800
600
400
200
0
cpm
H Molecular weight 93,000
800
600
400
200
0
cpm
0
20
40
60
5
10
20
30
seconds
minutes
○-control, ●-cyclic AMP (10^{-5}M)

3:2:9:4 The Phosphorylation of Specific Proteins in Subcellular Fractions Soluble (Sf) and Particulate (Pf)

Although it was shown that intrinsic cyclic AMP-dependent protein phosphorylation occured in the Pf, (see Figure 47) a few experiments were carried out in which crude homogenates were incubated with [γ^{32}P] ATP prior to preparing Sf and Pf, (Section 2:5:4:2). Figure 46 shows the pattern of protein phosphorylation in the crude homogenate and 10,000 g supernatant, 37,000 g supernatant and 37,000 g pellet prepared from it. The pattern of protein phosphorylation of the Sf prepared at 10,000 g was identical to that prepared at 37,000 g indicating that it was probably not due to particulate material which remained in suspension. Much less protein phosphorylation occured in the Pf (37,000 g pellet). The most intensely phosphorylated cyclic AMP-dependent protein was that at M_r 39,000 which was also present in the Sf prepared at 10,000 g and 37,000 g.

An inherent disadvantage in studying specific protein phosphorylation of the Sf and Pf prepared after reaction of the crude homogenate with [γ^{32}P] ATP was the time taken for centrifugation. The time course of protein phosphorylation in crude homogenates indicates that a maximum is achieved between 1 and 5 minutes, (Figure 44 and 45). The time taken to prepare Sf and Pf from the addition of [γ^{32}P] ATP to crude homogenates to solubilization of the proteins was 14 minutes for 10,000 g centrifugations and over 20 minutes for 37,000 g centrifugations. This would result in considerable dephosphorylation, consequently, in all subsequent experiments intrinsic protein phosphorylation of

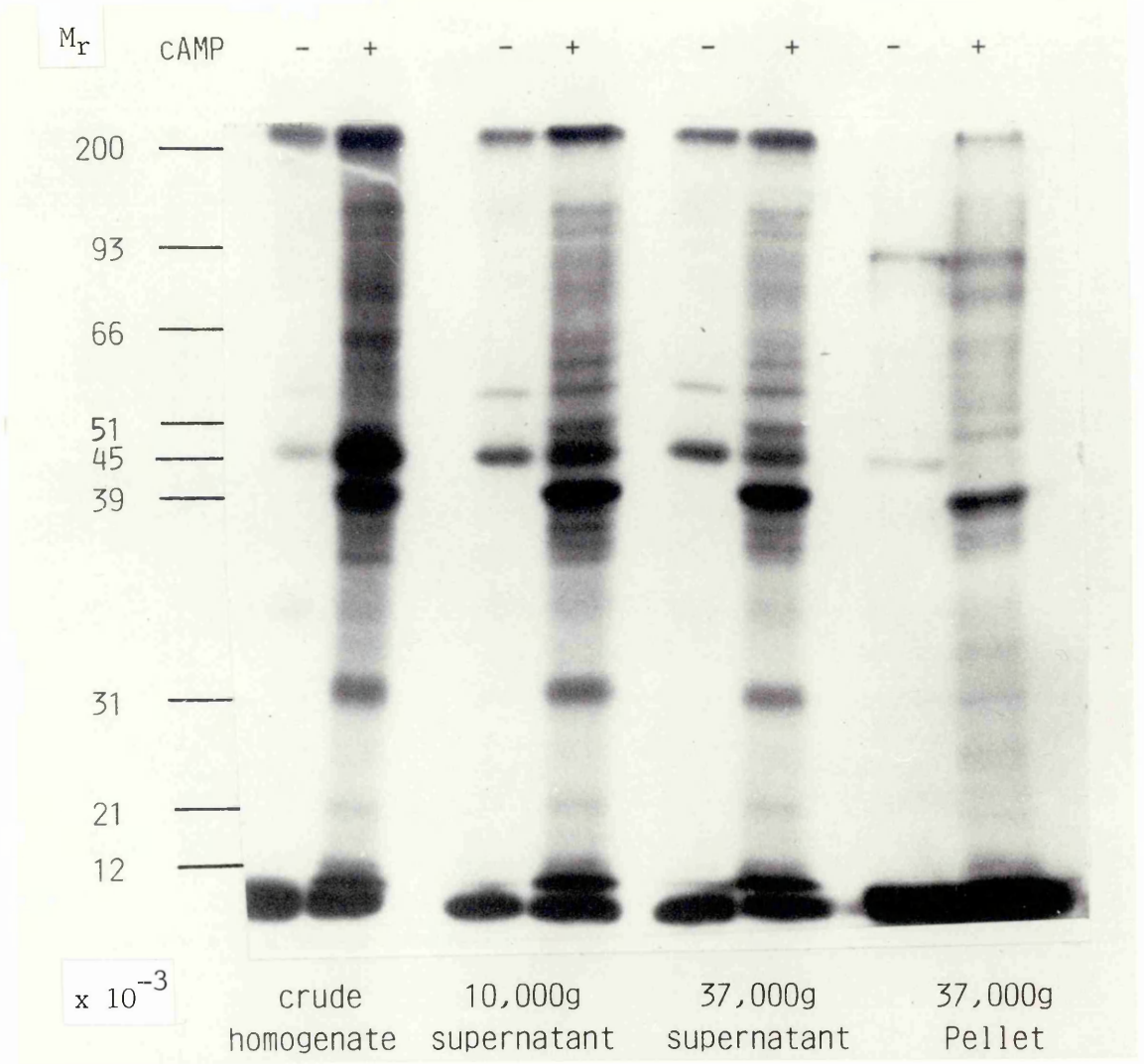


Figure: 46 The effect of cyclic AMP on the phosphorylation of specific proteins from the Sf and Pf prepared after incubation of crude homogenate with [γ^{32}P] ATP. Homogenates of cerebral ganglia were incubated with [γ^{32}P] ATP in the absence (-) or presence of 10^{-5} M cyclic AMP (+) prior to separation into Sf (10,000 g and 37,000 g supernatants) and Pf (37,000 g pellet), which were then electrophoresed on 10% polyacrylamide/SDS gels, (Section 2:5:4:2). Figure shows autoradiograph of gel.

the Sf and Pf was studied, (the Sf and Pf were themselves incubated with [γ^{32}P] ATP after preparation). Similar results were obtained by this method, (see Figure 47).

Figure 47 shows the effect of calcium and cyclic AMP on the phosphorylation of specific proteins from the crude homogenate, Sf and Pf. Much less cyclic AMP-dependent protein phosphorylation occured in the Pf than in the crude homogenate or the Sf, (see also Figures 48, 49 and 50). Phosphorylation of the M_r 39,000 protein in the presence of cyclic AMP persisted in all three fractions. Cyclic AMP also appeared to increase the incorporation of ^{32}P into a protein(s) at the top of the gel ($M_r \geq 200,000$) in all three fractions, but this was not observed in every experiment, (compare Figure 47 to Figures 48, 49 and 50).

$CaCl_2$ (1,000 µM) in the presence of EGTA (700 µM) stimulated an increased incorporation of ^{32}P into 3 proteins (M_r 62,000; 54,000 and 31,000) in the crude homogenate, (Figures 47 and 48). Calcium alone did not appear to affect the phosphorylation of any proteins in the Sf or Pf, (Figure 47). A very slight stimulation of a protein (M_r 54,000) was,however, observed (Figure 49).

Calmodulin (10 µg/ml) alone did not affect the phosphorylation of any proteins from the crude homogenate, Sf or Pf, (Figures 48,49 and 50). Calmodulin in the presence of calcium did not have any additional effect on the phosphorylation of proteins from the crude homogenate or the Sf in the presence of calcium alone. Calcium and calmodulin together did, however, stimulate

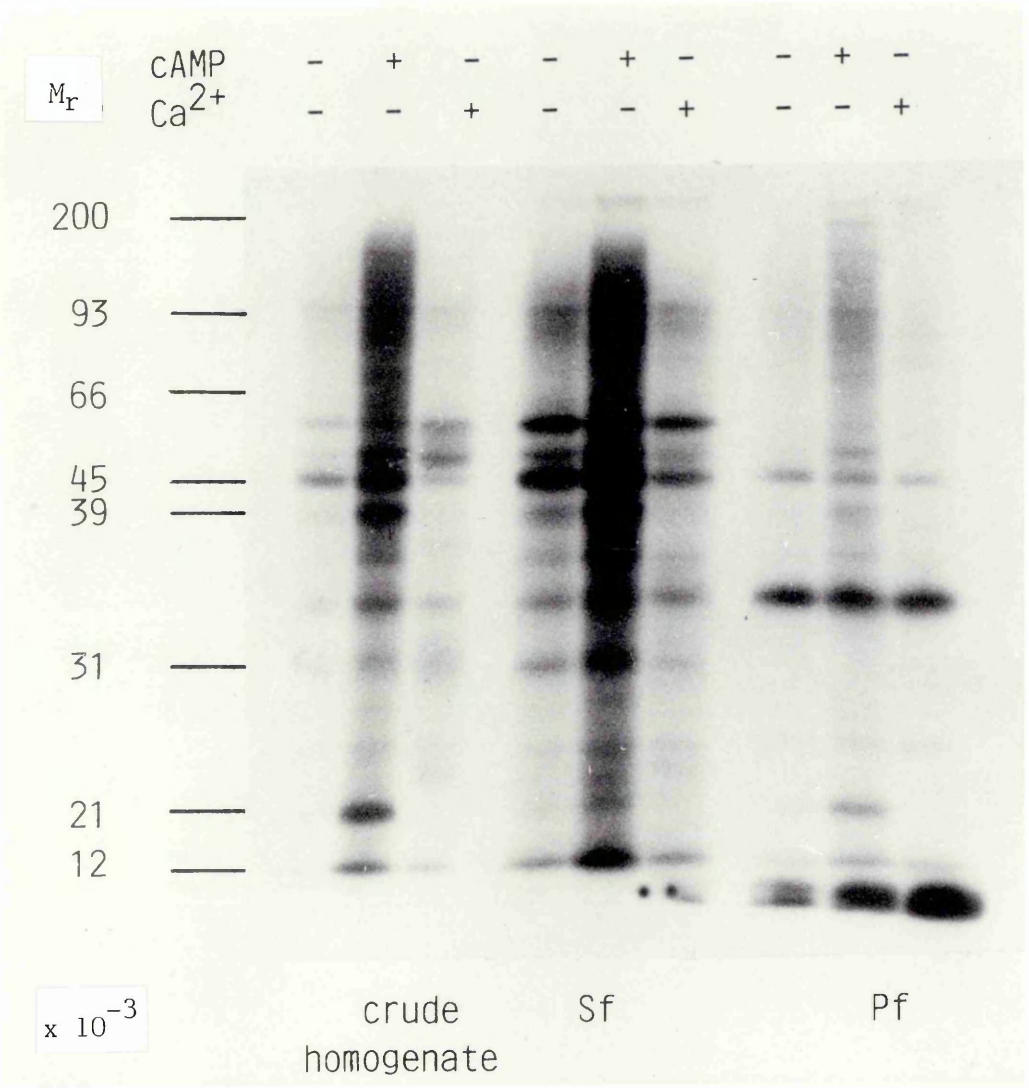


Figure: 47 The effect of cyclic AMP and calcium on the phosphorylation of specific proteins from the Sf and Pf. Tissue fractions prepared as described in Section 2:5:1:1 were incubated with [γ^{32}P] ATP for 1 minute in the absence (-) or presence (+) of either 10^{-5} M cyclic AMP or 1 mM $CaCl_2$ (all in the presence of 0.7 mM EGTA) followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:3). Figure shows autoradiograph of gel.

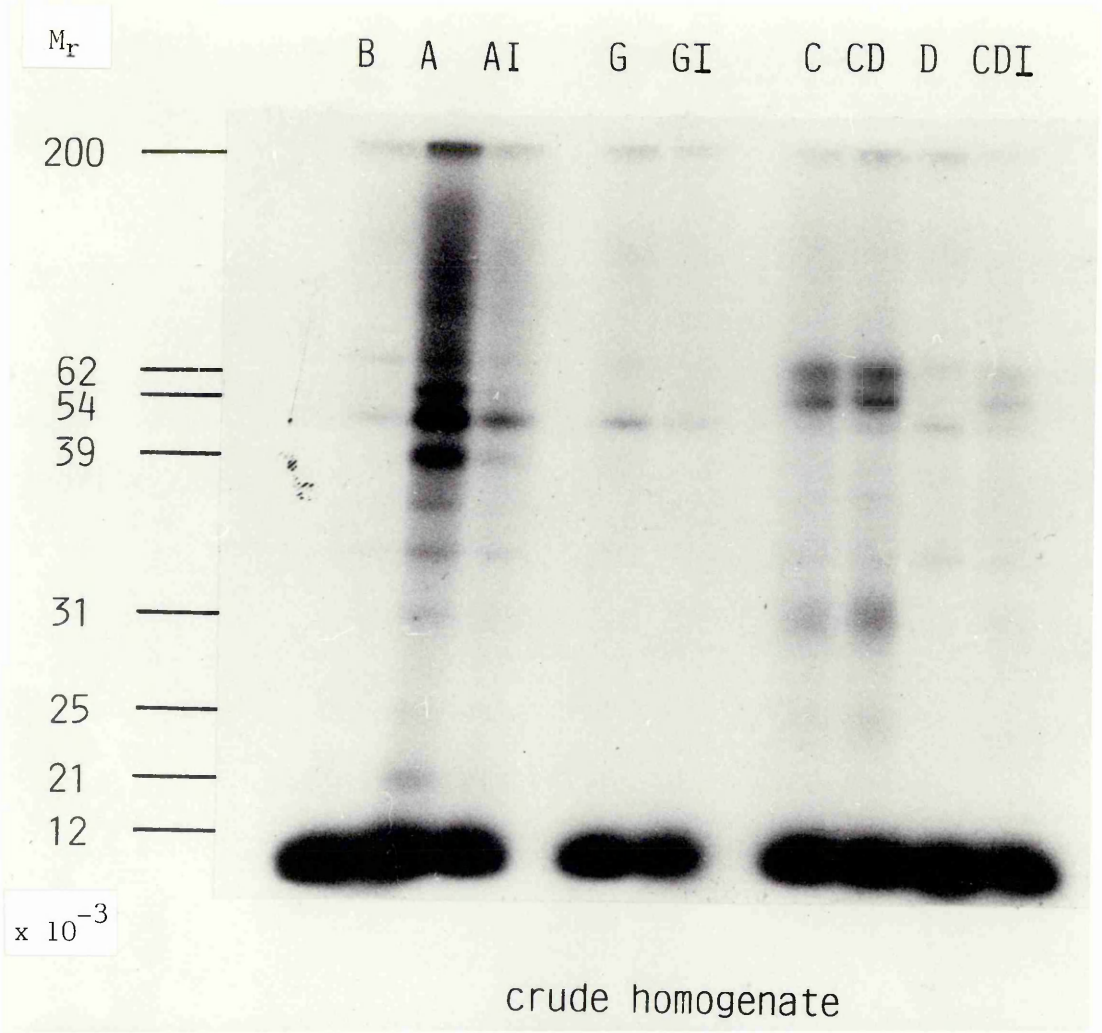


Figure: 48 The effect of cyclic AMP, cyclic GMP, calcium/calmodulin and protein kinase inhibitor on specific protein phosphorylation in homogenates of cerebral ganglia. Homogenates were incubated with [γ^{32}P] ATP with the following additions; buffer only (B), 10^{-5} M cyclic AMP alone (A) or with 20 µg/ml protein kinase inhibitor (AI), 10^{-5} M cyclic GMP alone (G) or with 20 µg/ml protein kinase inhibitor (GI), 1 mM $CaCl_2$ alone (C) or with 10 µg/ml calmodulin (CD), calmodulin alone (D) or with $CaCl_2$ and protein kinase inhibitor (CDI). Solubilizing buffer was added after incubating for 1 minute followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:3). Figure shows autoradiograph of gel.

EGTA (0.7 mM) was present in all incubations.

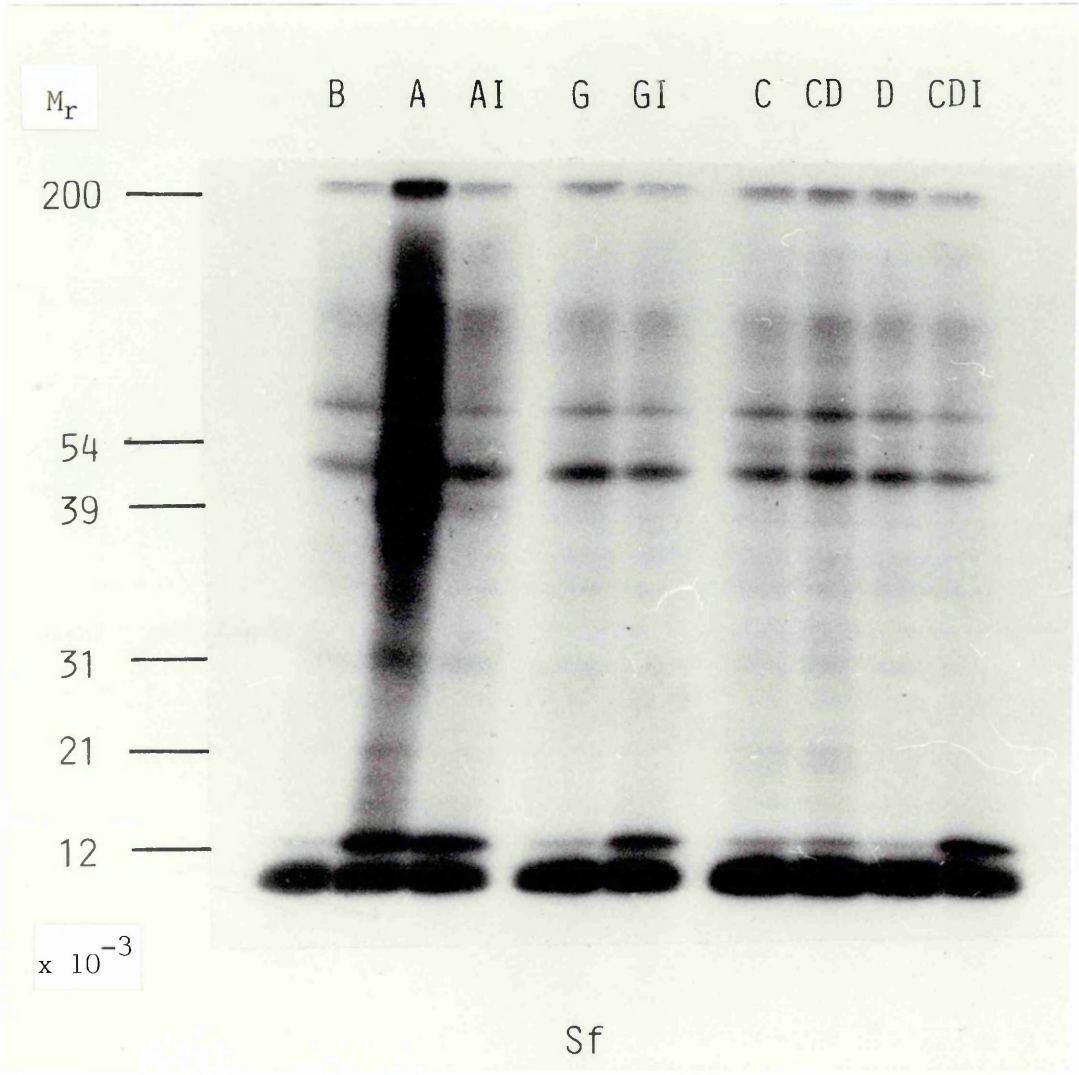


Figure: 49 The effect of cyclic AMP, cyclic GMP, calcium/calmodulin and protein kinase inhibitor on specific protein phosphorylation in the Sf. Aliquots of the Sf prepared as in Section 2:5:1:1 were incubated with [γ^{32}P] ATP with the following additions; buffer only (B), 10^{-5}M cyclic AMP alone (A) or with 20 µg/ml protein kinase inhibitor (AI), 10^{-5}M cyclic GMP alone (G) or with 20 µg/ml protein kinase inhibitor (GI), 1 mM $CaCl_2$ alone (C) or with 10 µg/ml calmodulin (CD), calmodulin alone (D) or with $CaCl_2$ and protein kinase inhibitor (CDI). Solubilizing buffer was added after incubating for 1 minute followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:3). Figure shows autoradiograph of gel.
EGTA (0.7 mM) was present in all incubations.

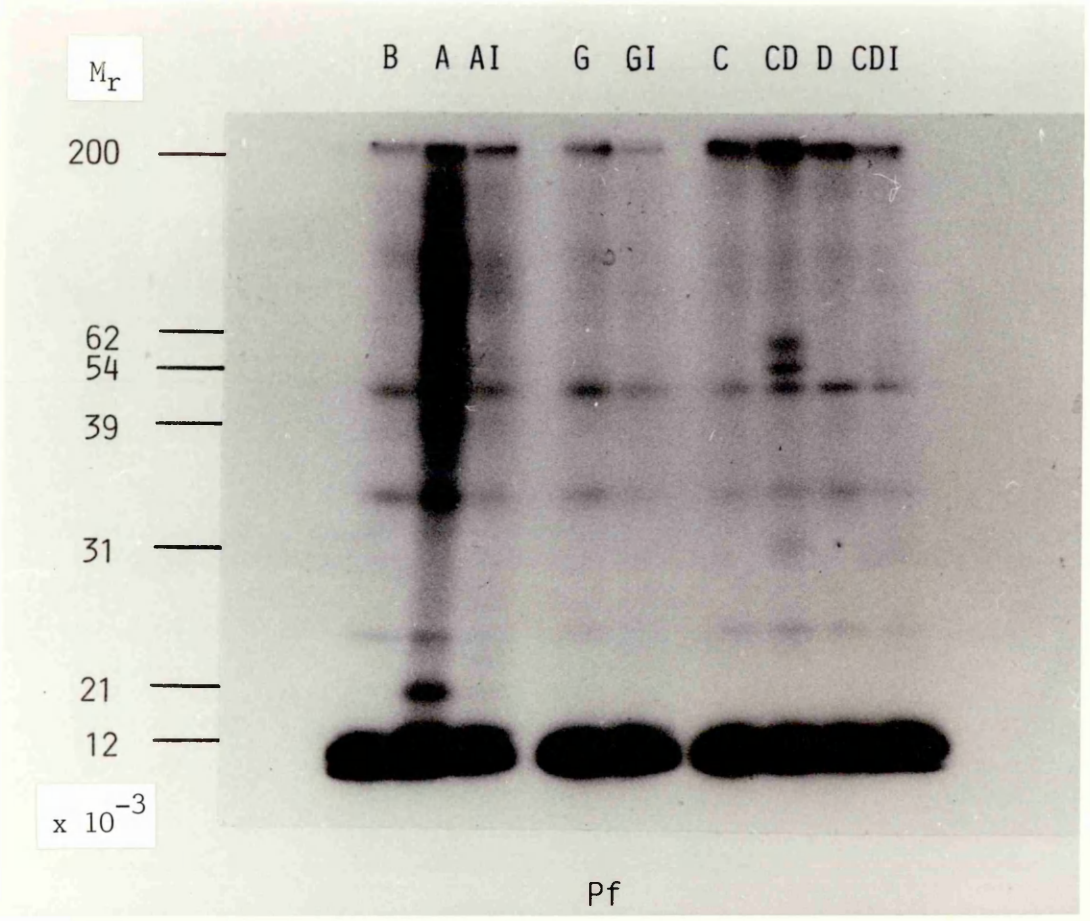


Figure: 50 The effect of cyclic AMP, cyclic GMP, calcium/calmodulin and protein kinase inhibitor on specific protein phosphorylation in the Pf. Aliquots of the Pf prepared as in Section 2:5:1:1 were incubated with [γ^{32}P] ATP with the following additions; buffer only (B), 10^{-5} M cyclic AMP (A) alone or with 20 µg/ml protein kinase inhibitor (AI), 10^{-5} M cyclic GMP alone (G) or with 20 µg/ml protein kinase inhibitor (GI), 1 mM $CaCl_2$ and protein kinase inhibitor (CD), calmodulin alone (D) or with $CaCl_2$ and protein kinase inhibitor (CDI). Solubilizing buffer was added after incubating for 1 minute followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:3). Figure shows autoradiograph of gel.
EGTA (0.7 mM) was present in all incubations.

the phosphorylation of 3 proteins (M_r 62,000; 54,000 and 31,000) in the Pf (Figure 50).

Cyclic GMP (10^{-5} M) increased the incorporation of ^{32}P into only one protein (M_r 45,000). This occured in all three fractions (Figures 48, 49 and 50).

Cyclic AMP-dependent protein kinase inhibitor[1] (20 µg/ml) prevented the cyclic AMP-dependent phosphorylation of all proteins from the crude homogenate, Sf and Pf. It also prevented the calcium stimulated phosphorylation of proteins from the crude homogenate and Sf and calcium/calmodulin stimulated protein phosphorylation in the Pf. It did not prevent the cyclic GMP stimulated phosphorylation of the M_r 45,000 protein from the Sf, although it did prevent the cyclic GMP-dependent phosphorylation of the M_r 45,000 protein from the crude homogenate and the Pf, (Figures 48, 49 and 50). Radioactivity was incorporated into the inhibitor protein itself, although this is only clearly visible in Figure 54 where the inhibitor protein migrated with the tracking-dye ($M_r \leq 12,300$).

[1]protein kinase inhibitor was prepared by the method of Walsh et al, (1971).

3:2:9:4b The Effect of Cyclic AMP on the Phosphorylation of Specific Proteins in Subcellular Fractions prepared in 0.25 M Sucrose

The pattern of radioactivity from locust nervous tissue which was homogenized in 0.25 M sucrose and incubated with [γ^{32}P] ATP, (Figure 51) was similar to that in which the tissue was homogenized in 6 mM Tris/maleate, (Figure 44).

The effect of cyclic AMP on the phosphorylation of specific proteins in subcellular fractions of locust cerebral ganglia was compared to its effect on that of rat cerebral cortex. The pattern of specific protein phosphorylation from homogenates of rat cerebral cortex was different than from homogenates of locust cerebral ganglia. Two main differences were observed:

1) Basal phosphorylation was higher i.e. more radioactive bands were observed in control incubations.
2) Cyclic AMP (10^{-5} M) did not affect the incorporation of ^{32}P into as many protein bands.

The effect of cyclic AMP on the phosphorylation of specific proteins in 25,000 g fractions was also studied. The cyclic AMP-dependent incorporation of ^{32}P into proteins from the 25,000 g supernatant of locust nervous tissue homogenate was similar to that of the crude homogenate. There were a greater number of phosphorylated proteins in control preparations from the 25,000 g supernatant of rat cerebral cortex homogenate compared with that of locust and cyclic AMP increased the phosphorylation of only 2 proteins (M_r 160,000 and 25,000). Cyclic AMP also increased the phosphorylation of many proteins in the 25,000 g pellet from locust nervous tissue homogenate. Only 3 proteins

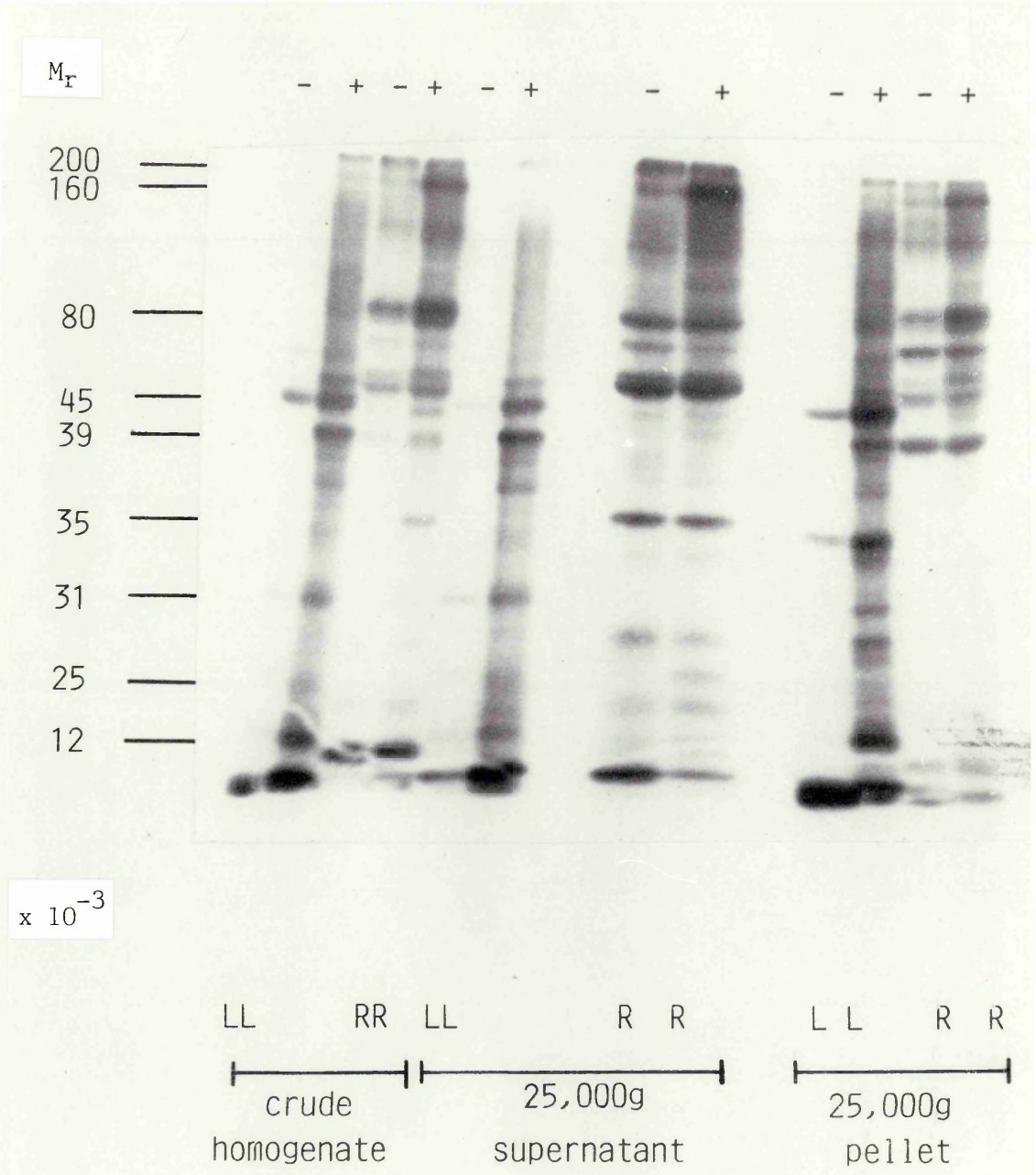


Figure: 51 Comparison of the effect of cyclic AMP on specific protein phosphorylation in tissue fractions from rat cerebral cortex and locust cerebral ganglia. Tissue fractions from the rat (R) and locust (L) prepared as described in Section 2:5:1:2 were incubated with [γ^{32}P] ATP in the absence (-) and presence of 10^{-5} M cyclic AMP (+) for 1 minute followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:3). Figure is autoradiograph of gel.

were affected by cyclic AMP in the 25,000 g pellet from rat cerebral cortex homogenate; M_r 160,000 and the two proteins reported by Ueda *et al*, (1973) M_r 80,000 and 48,000-50,000.

It appears that cyclic AMP increases the incorporation of ^{32}P into a much greater number of proteins from locust than from rat nervous tissue in all the fractions studied. The results also indicate that much less basal phosphorylation occurs in locust nervous tissue.

Figure 52 shows a comparison between cyclic AMP-dependent protein phosphorylation in 25,000 g and 150,000 g fractions from locusts.Similar patterns of radioactivity were observed in the 25,000 g and 150,000 g supernatants in the presence and absence of cyclic AMP. The only difference appeared to be that cyclic AMP increased the phosphorylation of a protein (M_r 35,000)in the 25,000 g supernatant but not in the 150,000 g supernatant. The 25,000 g pellet displayed a slightly different pattern of phosphorylation than either the 25,000 g or 150,000 supernatants. Cyclic AMP did not increase the incorporation of ^{32}P into the M_r 30,000, 51,000 or 64,000 proteins. Cyclic AMP did, however, increase the phosphorylation of a protein (M_r 21,000) in the 25,000 g pellet.

Samples of the 150,000 g pellet, usually termed the microsomal fraction, were examined by electron microscopy. Electron micrographs suggested that this fraction was composed of polysome material, (Figure 53). Many more phosphorylated proteins

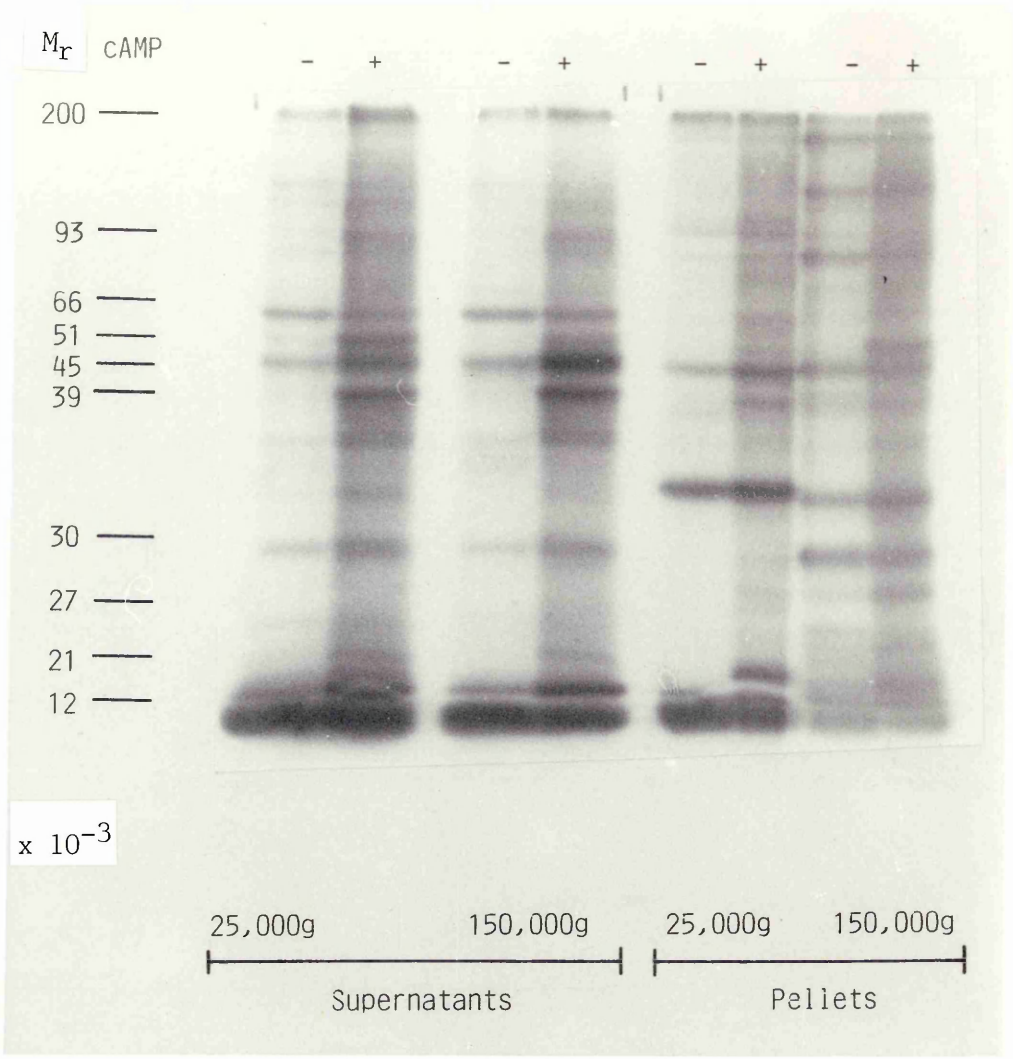


Figure: 52 The effect of cyclic AMP on specific protein phosphorylation in 25,000 g and 150,000 g fractions from locust cerebral ganglia. Tissue fractions prepared as in Section 2:5:1:2 were incubated with [γ^{32}P] ATP in the absence (-) or presence of 10^{-5} M cyclic AMP (+) for 1 minute followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:3). Figure shows autoradiograph of gel.

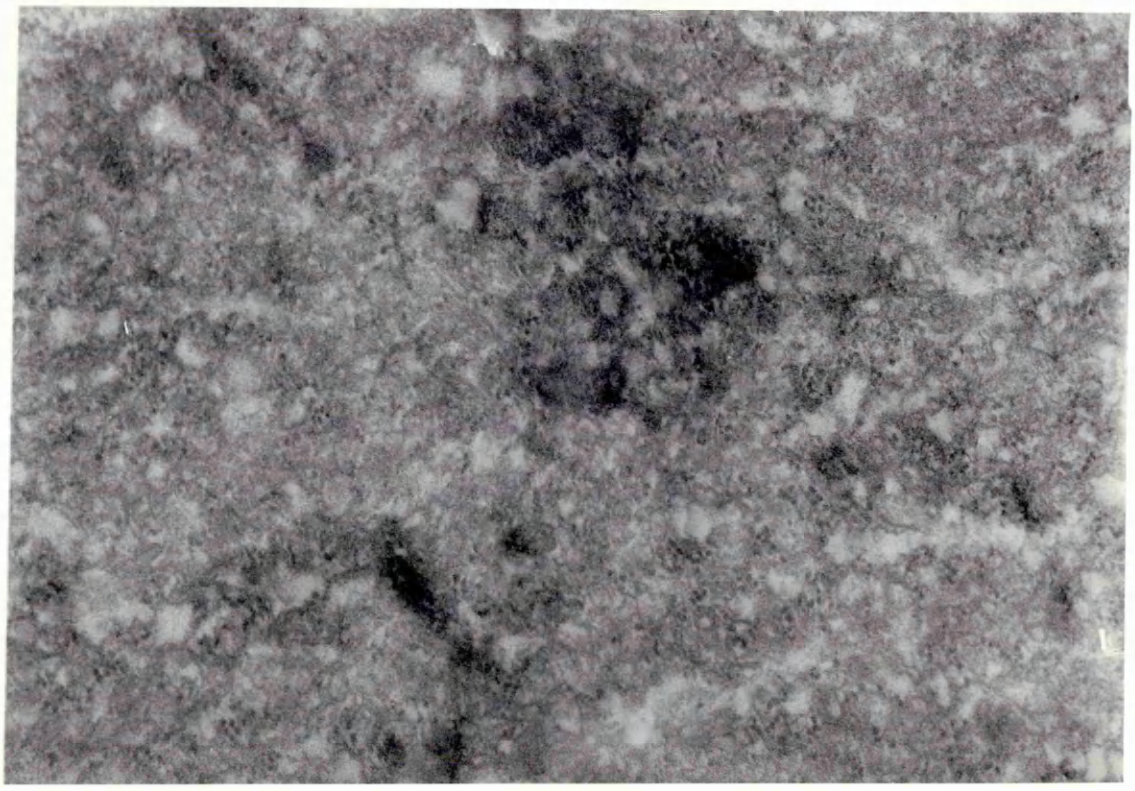


Figure: 53 Electron micrograph of the 150,000 g pellet, (Section 2:5:1:2) showing polysome-like material.

(Magnification x 30,000)

were present in the 150,000 g pellet, in the absence of cyclic AMP, than all the other fractions studied. Cyclic AMP stimulated an increased incorporation of ^{32}P into only three proteins (M_r 51,000; 27,000 and 21,000). Cyclic AMP did not stimulate an increased phosphorylation of the M_r 39,000 protein as in all the other fractions studied.

Specific protein phosphorylation was also studied in synaptosomes prepared as described in section 2:5:1:2 by a method similar to that of Breer, (1981), in which the 25,000 g pellet was resuspended in Ficoll and re-centrifuged. The material from the 25,000 g pellet separated into a pellicle, (floating at the top of the Ficoll) and a pellet. Samples of both the pellicle and pellet were examined by electron microscopy. Electron micrographs revealed that the pellicle contained synaptosome-like structures similar to those reported by Breer, (1981), i.e. membrane-bound structures containing mitochondria and numerous vesicles, (Figure 54). The pellet contained many free mitochondria, (Figure 55).

Figure 56 shows the pattern of phosphorylated proteins in the crude homogenate, mitochondrial fraction, synaptosomal fraction and the pellet/pellicle supernatant. The radioactive pattern from the crude homogenate and synaptosomal fraction were similar, for both control and cyclic AMP preparations. The incorporation of radioactivity into proteins from the mitochondrial fraction was much higher than either the crude homogenate or synaptosomal fraction. A greater number of proteins were phosphorylated

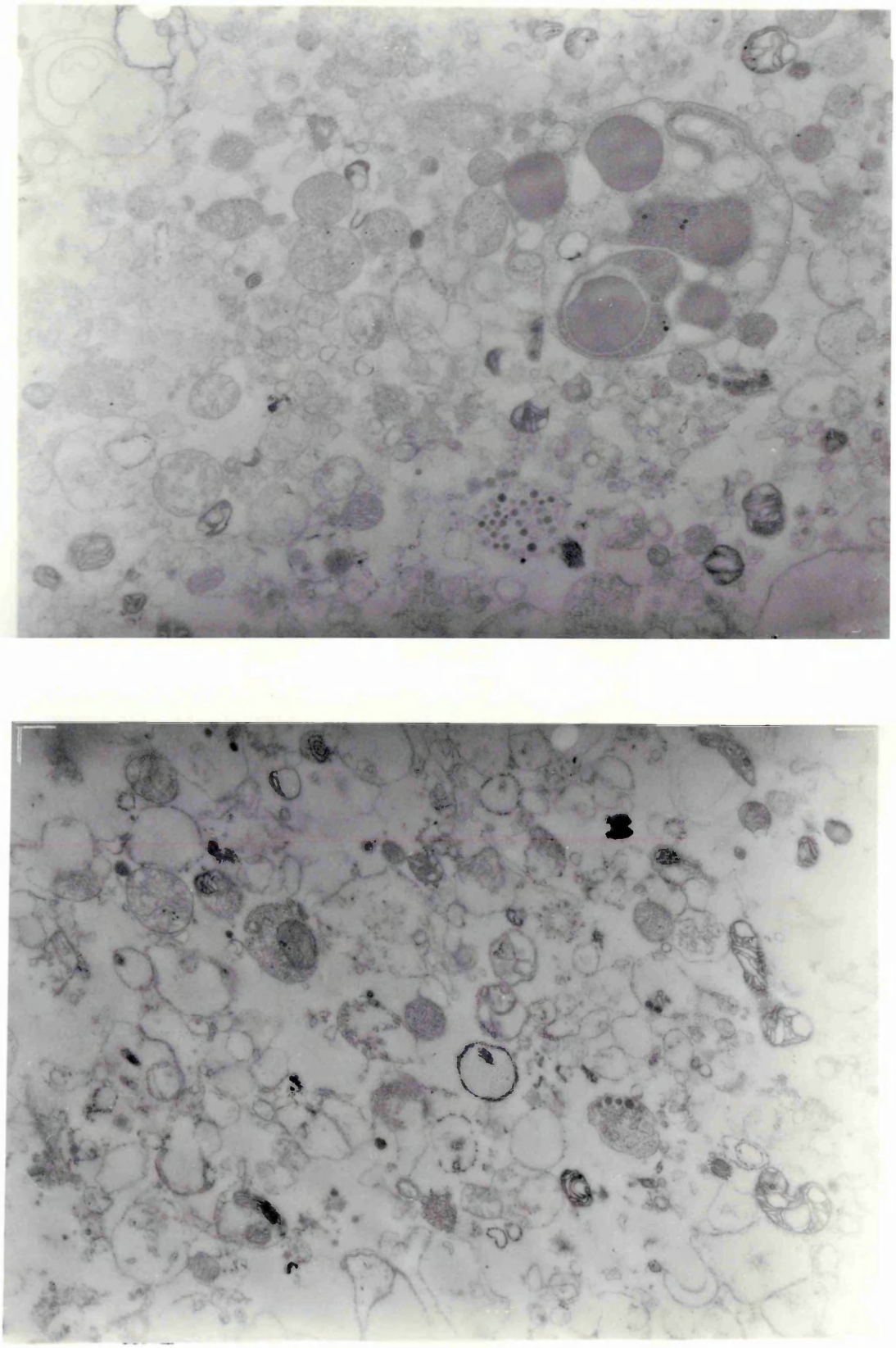


Figure: 54 Electron micrographs of the Ficoll floatation pellicle, (Section 2:5:1:2).

(Magnification both x 10,000)

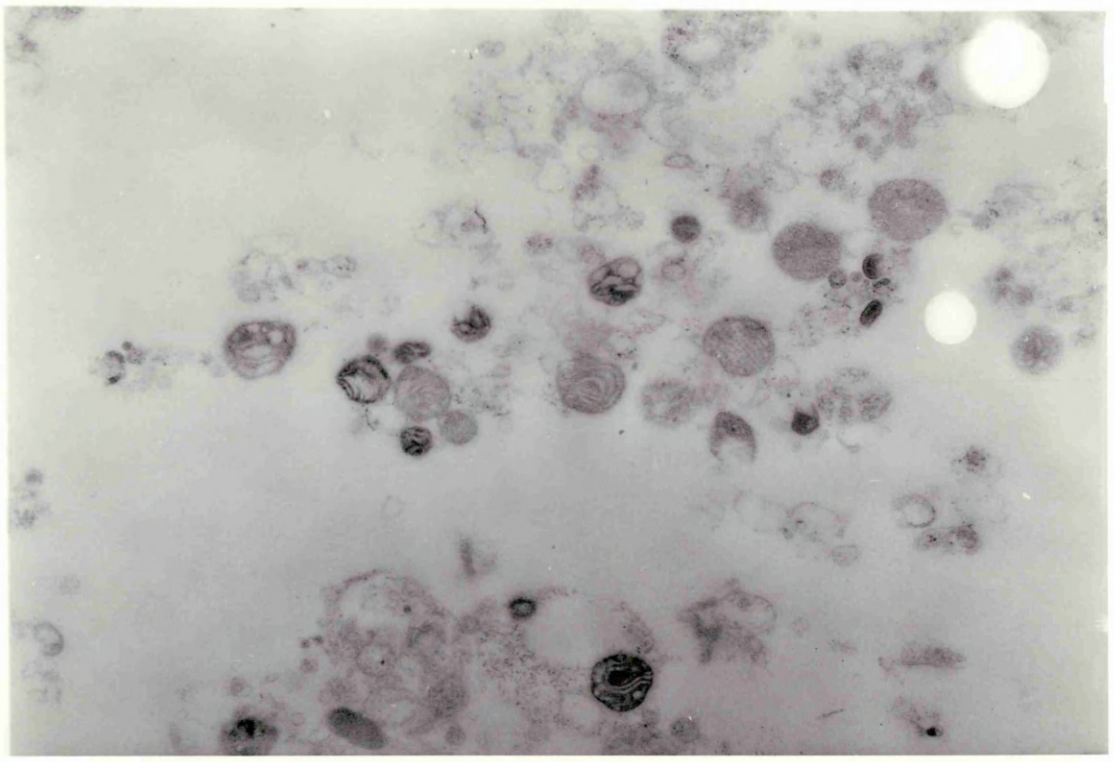


Figure: 55 Electron micrograph of the Ficoll pellet, (Section 2:5:1:2) showing well preserved free mitochondria.

(Magnification x 10,000)

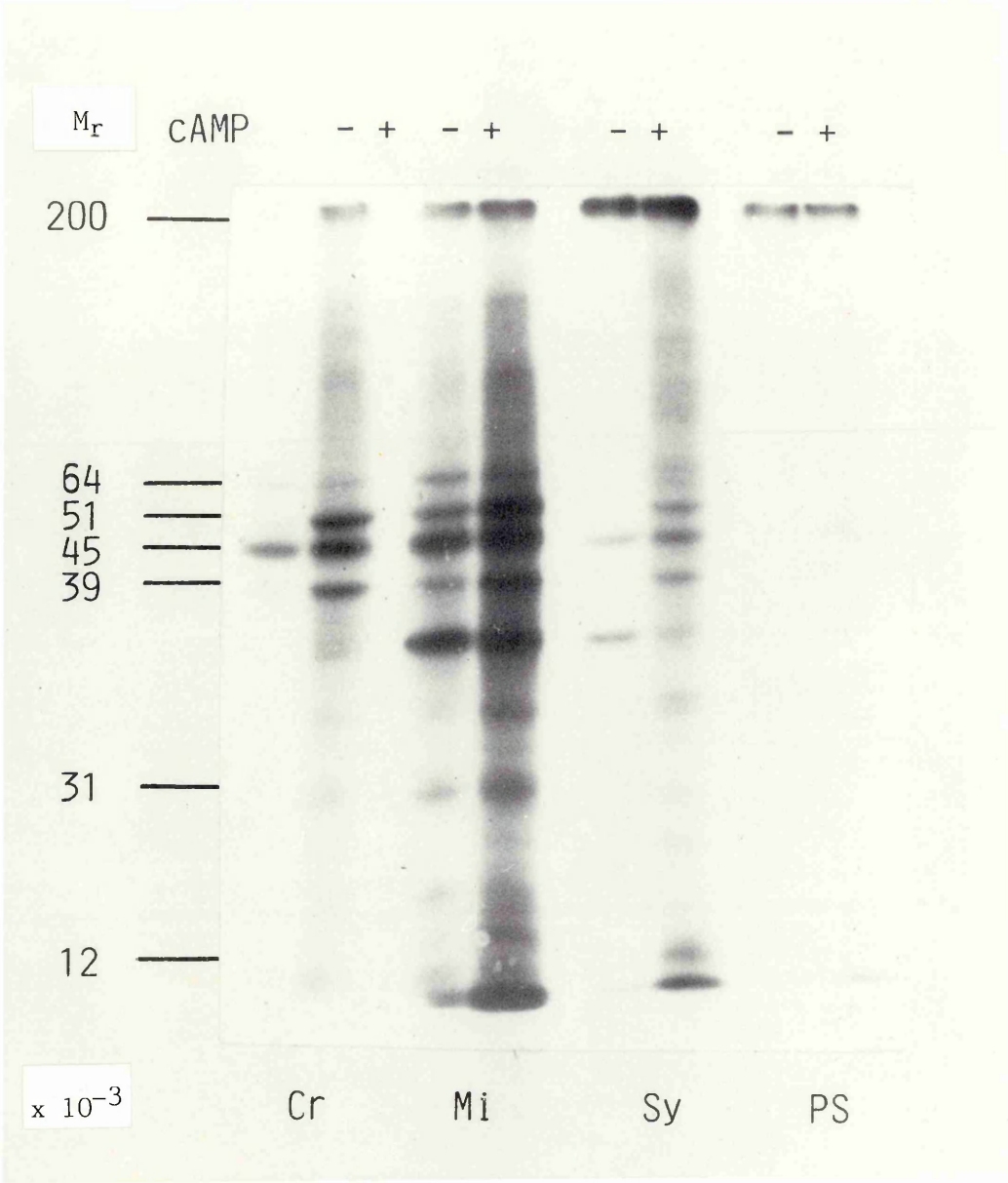


Figure: 56 The effect of cyclic AMP on specific protein phosphorylation in the synaptosomal and mitochondrial fractions. Aliquots of the crude homogenate (Cr), mitochondrial (Mi), synaptosomal (Sy), and pellicle/ pellet supernatant (PS) fractions from the centrifugation with Ficoll (Section 2:5:1:2) were incubated with [γ^{32}P] ATP in the absence (-) or presence of 10^{-5} M cyclic AMP (+) for 1 minute followed by electrophoresis on 10% polyacrylamide/SDS gels, (Section 2:5:3). Figure shows autoradiograph of gel.

in control incubations. Cyclic AMP did, however, increase the incorporation of ^{32}P into these proteins and other proteins.

No radioactive bands were detected from the pellicle/pellet supernatant either from control or cyclic AMP incubations, except for a small band of radioactivity at the top of the gel $\geq$ 200,000 daltons. Staining of the gels with Coomassie brilliant blue did not reveal any bands, except for a slight staining near the top of the gel. This indicated that the pellicle/pellet supernatant contained little or no protein.

The main aim of this thesis was to study the effect of octopamine, cyclic AMP and, to a lesser extent, calcium on the phosphorylation of protein from the CNS of S. gregaria. This discussion consists of an assessment of the methods used to study protein phosphorylation followed by discussion of the results obtained in relation to those obtained by other workers from both invertebrate and mammalian nervous tissue.

4:1 Inorganic ^{32}P Orthophosphate and the study of Protein Phosphorylation in Intact Ganglia

This section will be concerned with the study of the phosphorylation of total protein. The phosphorylation of specific protein will be discussed in section 4:3.

4:1:1 Phosphate Uptake

Inorganic radioactive phosphate, mainly the ^{32}P isotope and occasionally ^{33}P has been used extensively to study phosphorylation in vivo and in vitro. In one of the earliest in vivo studies with ^{32}P it was observed that after intraperitoneal injection or oral administration of [^{32}P] sodium phosphate, ^{32}P was absorbed into various tissues from rats including brain, (Cohn & Greenberg, 1938). Penetration of injected [^{32}P] phosphate into the brain from the blood-stream is, however, very slow due to the presence of the blood-brain barrier and equilibrium between plasma and brain takes almost 7 days to achieve in mice, (Dziewiatkowski & Bodian, 1950).

Another method of studying phosphorous metabolism is an in vitro preparation, where the intact tissue, or slices of it are placed

in a suitable supporting medium containing radiolabelled phosphate. This method was used in the present study.

The entry of phosphate into cells appears to occur by active transport rather than a passive diffusion through the membrane. At low temperature (7 ^{o}C) there is a very slow penetration of phosphate into erythrocytes, but very active penetration at 38 ^{o}C. This difference is much greater than would be expected on the basis of simple diffusion and agents which interfere with glycolysis such as iodoacetate, arsenate and fluoride also inhibit phosphate uptake, (Greenberg, 1952). The entry of ^{32}P through the axolemma of squid giant axon is inhibited by ouabain and markedly reduced by the removal of extracellular potassium, (Caldwell & Lowe, 1970). This suggests that the entry of orthophosphate is linked to the sodium pump. The rate of ^{32}P influx does not change if the extracellular phosphate concentration is increased from 0.1 to 0.8 mM, however, influx sharply increases with extracellular concentrations greater than 1 mM, (Caldwell & Lowe, 1970). It has also been shown by Gerlach et al, (1964) that iodoacetate, arsenate and fluoride have no effect on the entry of ^{32}P into erythrocytes with an extracellular phosphate concentration greater than 1 mM. In view of these observations it has been proposed by Caldwell & Lowe, (1970) that there are 2 mechanisms by which orthophosphate entry occurs;

1) Active transport linked to metabolism and operating at extracellular phosphate concentrations less than 1 mM and directly affected by ouabain and metabolic inhibitors.

2) Passive entry at extracellular concentrations greater than 1 mM, possibly through the same channels by which phosphate efflux occurs and would therefore be insensitive to metabolic inhibitors.

In the present study the extracellular phosphate concentration even with the highest amount of ^{32}Pi (170 μCi/ml) would have been far lower than 1 mM,as cerebral ganglia were incubated with carrier-free ^{32}Pi in the absence of any added unlabelled phosphate. Therefore if the influx of phosphate into S. gregaria nervous tissue occurs by similar processes to that of squid giant axon then the entry of phosphate would have occured by active transport.

4:1:2 Measurement of Phosphate bound to Protein

After the incubation of tissues with ^{32}Pi a common procedure is to homogenize the tissue with either perchloric acid or trichloroacetic acid. Radioactivity is found to be incorporated into both acid soluble and insoluble material. The acid soluble fraction from mammalian brain incubated with ^{32}Pi has been shown to contain radioactivity incorporated into; ATP, ADP, GTP, GDP, phosphocreatine, NADP, hexose monophosphates and orthophosphate, (Heald, 1960). After the removal of compounds soluble in acid, the residual ^{32}P is contained in a mixture of compounds collectively termed 'acid insoluble phosphates'. In cerebral slices of guinea-pig brain incubated with ^{32}Pi,radioactivity in the acid insoluble fraction is incorporated into proteins, phospholipids and nucleic acids, (Heald, 1957).

In the present study the TCA precipitated homogenate of cerebral ganglia was washed twice with TCA to remove acid soluble radio-

activity from the tissue residue. Three consecutive extractions with TCA removed almost all of the soluble radioactivity, (very little is removed in the last wash). This was in agreement with the findings of Heald, (1957) who found that in homogenates of guinea-pig cerebral cortex, 3 extractions with TCA were sufficient to remove soluble ^{32}P. Subsequent TCA washes did not remove any further radioactivity. Problems in removing contaminating radioactivity have been reported when ^{32}P of high specific activity is used. Davidson et al, (1951) reported that traces of ^{32}P were removed even after 50 extractions with TCA, which was attributed to traces of adsorbed ^{32}P. Continued TCA extraction could, however, result in a slow breakdown of the acid insoluble ^{32}P, (Heald, 1957).

In order to measure the phosphate bound to protein it is necessary to remove nucleic acids and phospholipids from the acid insoluble residue. In slices of guinea-pig brain incubated with ^{32}Pi for as short a time as 3 minutes, 35.6% of the radioactivity present in the acid insoluble residue is incorporated into phospholipids and 5.2% into nucleic acids. Only 38% of the total acid insoluble radioactivity is incorporated into protein, (Heald, 1957). Similarly in invertebrate nervous tissue a large proportion of the acid insoluble radioactivity is present as phospholipid and nucleic acid. In Aplysia abdominal ganglia incubated with ^{32}Pi for 22 hours, 20% of the total acid insoluble radioactivity was incorporated into phospholipids and 40% into nucleic acids, (Levitan & Barondes, 1974). In S. gregaria cerebral ganglia also incubated for 22 hours with ^{32}Pi,

15.1% of the acid insoluble radioactivity was removed by acidified chloroform/methanol and 60.4% was incorporated into RNA, (Figure 15).

The most popular organic solvents employed to extract lipids from fractionated tissue have been ethanol/diethyl ether (1:1, v/v) and chloroform/methanol, (2:1, v/v). The effectiveness of these 2 solvent mixtures was assessed by Folch et al, (1951), who found that ethanol/ether does not extract all the lipids present in nervous tissue, and the extract contains substances other than lipid. Chloroform/methanol is more rigorous in removing total lipid from acid insoluble residues, (Folch & Lees, 1951). Chloroform/methanol does not, however, remove phosphoinositides which contribute greatly to the radioactivity remaining in the de-lipidized residue after the removal of nucleic acids. It has been reported that chloroform/methanol acidified with concentrated HCl (200:100:1 v/v respectively) readily removes phosphoinositides. The proportion of the total ^{32}P present after the removal of nucleic acids and extraction with acidified chloroform/methanol, which is incorporated into protein now approaches 80%, (Rodnight, 1971). The extraction of S. gregaria acid insoluble residue with acidified chloroform/methanol removed additional radioactivity indicating the removal of phospholipids and possibly phosphoinositides, (Figures 14 & 15) although some protein may also be removed with the chloroform/methanol, (Table 9).

Nucleic acids can be removed from the de-lipidized acid insoluble

residue by treatment with hot TCA or, alternatively, by treatment with ribonuclease. In the present study de-lipidized acid-insoluble residues from S. gregaria nervous tissue were treated with ribonuclease. This has been shown to be very effective in removing RNA. Ribonuclease treatment of the de-lipidized acid-insoluble residue from Aplysia nervous tissue incubated with [^{3}H] uridine for 22 hours, removes more than 90% of the [^{3}H] RNA, (Levitan & Barondes, 1974)

Most of the acid insoluble ^{32}P remaining after the removal of phospholipids and nucleic acid should be bound to protein. This is usually determined by measuring the alkali-labile radioactivity, where the protein residue is incubated with 1 M NaOH at 37 ^{o}C for up to 18 hours (12 minutes at 100 ^{o}C is equally effective). The protein is re-precipitated and the ^{32}P released by the alkali treatment represents that which was originally bound to protein, (Rodnight, 1971; Rodnight et al, 1975). An alternative method is to measure the amount of ^{32}P covalently bound to serine or threonine. This is achieved by hydrolyzing the extracted protein with 2 M HCl at 100 ^{o}C for 6 hours and separating the hydrolysate by high voltage paper electrophoresis to recover phosphoserine, (Rodnight, 1971; Rodnight et al, 1975). The latter method is more precise from a qualitative point of view as it circumvents errors due to non-covalently bound ^{32}P and alkali-labile ^{32}P not bound to protein. However, it is not representative of the total ^{32}P bound to protein and in a direct comparison the radioactivity recovered as phosphoserine amounts to about 30% of the alkali-labile radioactivity, (Rodnight et al, 1975). During acid

hydrolysis of the protein, phosphoserine breaks down and under the hydrolysis conditions described above, only 46.1% remains as phosphoserine, (Bylund & Huang, 1976), therefore, this method is only useful from a comparative point of view. The alkali-labile ^{32}P is thought to closely represent the ^{32}P bound to protein, and in ox brain, of the total radioactivity remaining in the acid-insoluble residue after the removal of phospholipids/phosphoinositides and nucleic acids, 80% is alkali-labile, (Rodnight, 1971).

In the present study the radioactivity present in the acid-insoluble residue after treatment with acidified chloroform/methanol and ribonuclease, (referred to as the 'washed' protein pellet) was taken as a measure of the ^{32}P bound to protein. The radioactivity recovered from polyacrylamide/SDS gels after electrophoresis of 'washed' protein pellets represented the ^{32}P bound to protein, as nucleic acids and phospholipids had been removed and ^{32}P not covalently bound to protein would have been eluted from the gel under the electrophoresis conditions employed in the present study. Of the total radioactivity from 'washed' protein pellets placed onto the gels, between 82% and 93% was recovered in the gels after electrophoresis (calculated from the data in Table 8). This indicates that between 82% and 93% of the total radioactivity present in 'washed' protein pellets was ^{32}P covalently bound to protein. In was concluded then that radioactivity present in 'washed' protein pellets from S. gregaria closely represented the ^{32}P bound to protein.

4:2 The Study of Protein Phosphorylation in Homogenized Tissue using [γ^{32}P] ATP

This section will be concerned with assessing the methods used to study the phosphorylation of total protein. Methods used to study specific protein phosphorylation using [γ^{32}P] ATP will be discussed in Section 4:3.

In measuring the total protein-bound ^{32}P from [γ^{32}P] ATP the acid-insoluble residue was extracted with acidified chloroform/methanol as described in Section 4:1 to ensure that no lipid-bound radioactivity was included in the final estimation of protein-bound ^{32}P. It is highly unlikely that in the short incubation times employed to study phosphorylation with [γ^{32}P] ATP in tissue fractions that ^{32}P would be incorporated into nucleic acids. Very little radioactivity was removed from the de-lipidized acid-insoluble residue from homogenates of S. gregaria nervous tissue after treatment with ribonuclease, (Table 9). In comparison, a large amount of radioactivity was released by ribonuclease treatment after 22 hours incubation of intact ganglia with ^{32}Pi, (Figure 15).

Treatment of the 'washed' protein pellet from S. gregaria with pronase removed almost 95% of the radioactivity present. This suggests that the radioactivity present in the acid-insoluble residue after treatment with acidified chloroform/methanol and ribonuclease actually was representative of the radioactivity associated with protein. The manner in which it is associated is, however, not clear. An appreciable amount of radioactivity

(in the order of 35% that of normal control incubations)was present in the 'washed' protein pellets from TCA denatured protein incubated with [γ^{32}P] ATP, (Figure 24). It is reasonable to assume that the addition of TCA to incubations containing tissue fractions before the addition of [γ^{32}P] ATP would denature both the kinases and acceptor proteins, therefore, it would not be possible for ^{32}P to be transferred and become covalently bound to protein via enzymic processes. A possible explanation is that the radioactivity is due to ^{32}P being adsorbed to the denatured protein although it does not seem possible that it would survive the extraction procedure. In addition, if it was due to adsorbed radioactivity, it could be either ^{32}Pi or [γ^{32}P] ATP. In tissues incubated with ^{32}Pi of high specific activity it has been reported that it may be possible for ^{32}P which is adsorbed to components of the acid-insoluble residue to survive up to 50 extractions with TCA (Davidson et al, 1951). It is thought that ^{32}P adsorbed to the acid-insoluble residue from homogenized tissues incubated with [γ^{32}P] ATP can still be present after extraction with acidified chloroform/methanol and hot TCA, (Rodnight et al, 1975: Weller, 1979). It has also been reported that a TCA-insoluble phosphorylated compound is formed non enzymically from a mixture of ATP, cyclic AMP, Mg^{2+} ions and F^- ions, (Greenaway, 1972). However, it does not appear that the radioactivity present in 'washed' protein pellets from TCA-denatured protein incubated with [γ^{32}P] ATP is due to the formation of a similar TCA insoluble-compound. The compound reported by Greenaway, (1972) had an absolute requirement for F^- ions which were not present.

In the present study it was intended to study changes in protein phosphorylation in response to various agents. These agents should only affect ^{32}P which is covalently bound to protein by enzymic processes. The quantity of protein present would be the same both in their absence and presence and therefore, also the amount of non-enzymically incorporated ^{32}P. Evidence in support of this explanation is provided by the observation that cyclic AMP (which did not affect the amount of protein recovered) increased the amount of ^{32}P incorporated into total protein from normal incubations but did not affect the level of ^{32}P from preparations where TCA denatured protein was incubated with [$\gamma^{32}P$] ATP, (Figure 24). It was concluded that the change in ^{32}P present (in 'washed'protein pellets) in response to various agents was a valid measure of the change in ^{32}P covalently bound to protein by enzymic processes.

4:3 Polyacrylamide/SDS Gel Electrophoresis and the Study of Phosphorylation of Specific Proteins

Phosphorylated proteins can be detected by first separating them by polyacrylamide/SDS gel electrophoresis and determining which of these contain covalently-bound phosphate.

A method has been described whereby phosphorylated proteins can be detected directly by a staining method. The proteins are separated on polyacrylamide/SDS gels which are incubated with NaOH in the presence of Ca^{2+} ions to form insoluble calcium phosphate, as phosphate is released from the protein. Gels are then treated with ammonium molybdate in dilute nitric acid to form nitrophosphomolybdate which is stained with methyl green. Green bands are obtained which correspond to proteins which originally contained alkali-labile phosphate. This method is capable of detecting 1 n mole of protein-bound phosphate, (Cutting & Rott, 1973). This method was used to see if phosphoproteins could be detected from crude homogenates of locust cerebral ganglia incubated with unlabelled ATP. No green bands were obtained in 2 experiments carried out under the conditions normally employed with radiolabelled ATP indicating that this method was not sufficiently sensitive under these conditions.

The most popular method of detecting phosphoproteins is by incubating either intact tissues with ^{32}Pi or homogenized tissues with [$\gamma^{32}P$] ATP, separating the proteins on polyacrylamide/SDS gels and detecting the radioactivity by autoradiography or slicing and scintillation counting, or a combination of both.

Incubations of homogenates with [γ^{32}P] ATP (after being homogenized in an appropriate buffer) are usually terminated directly with a solubilizing buffer containing SDS and 2-mercaptoethanol then boiled in preparation for electrophoresis. It is not necessary to remove free ^{32}Pi or [γ^{32}P] ATP as these move much faster than proteins on electrophoresis on polyacrylamide/SDS gels due to their small mass and high negative charge. ^{32}Pi or [γ^{32}P] ATP were never detected when electrophoresed in parallel with protein samples, although radioactivity could be detected in the lower well buffer.

Samples were not extracted with TCA for slab-gel electrophoresis as TCA-denatured protein from S. gregaria cerebral ganglia did not resolve very well. An SDS solubilizing buffer was added directly to the homgenized tissue, (Section 2:3:4). Consequently, lipids and nucleic acids were not removed from samples before electrophoresis. In both intact and homogenized preparations of S. gregaria ganglia, radioactivity from ^{32}Pi and [γ^{32}P] ATP respectively was removed by extraction with acidified chloroform/methanol, (Figures 14 & 15; Table 9). This indicates that some of the ^{32}P was bound to lipids. However, the solubilization of tissue samples in SDS buffers releases lipids from protein interactions and they migrate with/or ahead of the electrophoretic front on polyacrylamide/SDS gels, (Ramirez et al, 1972). Phospholipids from homogenized neuroblastoma cells and homogenates of Aplysia nerve roots incubated with [γ^{32}P] ATP, migrate more rapidly than the respective phosphoproteins when electrophoresed on 7-14% gradient polyacrylamide/SDS gels. Electrophoresis

of the chloroform/methanol extracted acid-insoluble residue from homogenates of S. gregaria cerebral ganglia incubated with [γ^{32}P] ATP revealed that the chloroform/methanol only selectively removed radioactivity ahead of the tracking-dye. This indicated that none of the radioactive bands resolved in the gel were due to ^{32}P bound to lipid. Although lipids have a lower molecular weight than the smallest proteins, they have been shown to behave like small proteins in polyacrylamide/SDS gels. Gangliosides labelled in vivo by injecting mice with [^{14}C] glucosamine migrate as macromolecules with an apparent molecular weight of 10,000 when electrophoresed on polyacrylamide/SDS gels and are diffusely stained by Coomassie blue, (Dutton & Barondes, 1972; Ramirez et al, 1972). Gangliosides, however, have not been shown to contain covalently bound phosphorous, (Brunngraber, 1979) and would, therefore, not be detected on autoradiography of electrophoresed tissues labelled with ^{32}P.

Intact ganglia incubated with ^{32}Pi for 22 hours incorporated a substantial amount of radioactivity into RNA, (Figure 14). In comparison, very little was incorporated after 3 hours, (Figure 15). Ribonucleic acids migrate as bands on polyacrylamide/SDS gels as they are polyanions and like proteins in SDS move toward the positive electrode, (Bhorjee & Pederson, 1976). In order to ensure that the radioactive bands on polyacrylamide/SDS gels are due to phosphoproteins and not ^{32}P bound to RNA, it is essential that RNA is removed. RNA can be removed from polyacrylamide/SDS gels by incubating with hot TCA. This treatment has been shown not to affect [^{14}C]-labelled

proteins from HeLa cells, or phosphorylated spectrin from human erythrocytes, (Bhorjee & Pederson, 1976). Treatment of the gels with hot TCA did not affect the radioactive bands (before the tracking-dye) obtained on electrophoresis of tissue from intact ganglia of S. gregaria incubated with ^{32}Pi for 3 hours, (Figure 20). However, hot TCA treatment of gels removed radioactive bands with apparent molecular weights of 40,000 and 48,000 when samples from ganglia incubated with ^{32}Pi for 22 hours were electrophoresed, (Figure 22). This indicated that these 2 bands were due to ^{32}P incorporated into nucleic acid as ganglia incubated for 22 hours with ^{32}Pi incorporated a large amount of ^{32}P into RNA, (Figure 15).

Homogenates of cerebral ganglia incubated with [$\gamma^{32}P$] ATP did not incorporate ^{32}P into RNA, (Table 9), consequently, these gels were not treated with hot TCA.

Incubation of [^{32}P] phosphoproteins from Aplysia nervous tissue with pronase prior to electrophoresis results in less than 2% of the radioactivity of controls remaining in gels after electrophoresis, (Levitan & Barondes, 1974). Similar results were obtained when the 'washed' protein pellets from homogenates of S. gregaria which had been incubated with [$\gamma^{32}P$] ATP, were treated with pronase. No radioactivity was detected in the gels except ahead of the tracking-dye, (Figure 43) indicating that the radioactive bands resolved in gels were due to protein-bound ^{32}P.

4:4 The Stimulation of Protein Phosphorylation by Octopamine

4:4:1 In intact tissues

Octopamine (10^{-4} M) stimulates the phosphorylation of a protein (M_r 120,000) from intact abdominal ganglia of Aplysia incubated with radiolabelled phosphate, (Levitan & Barondes, 1974). Dibutyryl cyclic AMP mimics the effect of octopamine and phentolamine blocks the octopamine stimulated phosphorylation. Octopamine stimulates an increase in the level of cyclic AMP in intact Aplysia abdominal ganglia which can be prevented in the presence of phentolamine, (Levitan et al, 1974). This suggests that the increase in phosphorylation (stimulated by octopamine) of the M_r 120,000 protein is mediated by cyclic AMP.

Octopamine stimulates the phosphorylation of a protein (M_r 39,000) from S. gregaria intact cerebral ganglia after 10 minutes and 22 hours. Two other proteins (M_r 21,000 and 25,000) are also stimulated after 22 hours, (Figures 21 and 22). Dibutyryl cyclic AMP stimulates the phosphorylation of the M_r 39,000 protein after 10 minutes, (Figure 21), although phosphorylation of at least 7 other proteins is also increased. This is not observed in Aplysia intact ganglia where dibutyryl cyclic AMP only stimulates the phosphorylation of the M_r 120,000 protein and does not affect the phosphorylation of any other protein, (Levitan & Barondes, 1974). Cyclic AMP has a similar effect on protein phosphorylation in homogenates of S. gregaria cerebral ganglia as dibutyryl cyclic AMP has on intact ganglia, (Figure 44 and 21). Octopamine increases the level of cyclic AMP in homogenates of cerebral ganglia, (Kilpatrick et al, 1980). It

appears then that the octopamine-stimulated protein phosphorylation in intact ganglia is mediated by cyclic AMP in S. gregaria also.

The octopamine-stimulated increase in the level of cyclic AMP in intact abdominal ganglia of Aplysia has been measured up to 22 hours. A two-fold increase is observed after 10 minutes which decreases to the control level over the next 50 minutes. After 22 hours a second increase in the cyclic AMP level occurs, (Levitan et al, 1974). The octopamine-stimulated phosphorylation of the M_r 120,000 protein was measured after 22 hours which coincides with the second increase in the cyclic AMP level, (Levitan & Barondes, 1974). Octopamine stimulates an increase in the phosphorylation of total protein from S. gregaria intact cerebral ganglia after 10 minutes which decreases to basal between 10 minutes and 6 hours. A second stimulation is observed after 22 hours, (Figure, 18). It was interesting to note that the time course of protein phosphorylation in the presence of octopamine in S. gregaria was similar to that of the increase in the level of cyclic AMP in response to octopamine in Aplysia (Levitan et al, 1974).

It is not clear if the secondary stimulation by octopamine of the total protein phosphorylation in S. gregaria and the increase in cyclic AMP level and phosphorylation of the M_r 120,000 protein in Aplysia after 22 hours,requires octopamine to be present throughout or whether a brief initial exposure is sufficient. The effect of removing octopamine after a brief initial exposure then continuing incubations in its absence was not studied. However, intact tissues from S. gregaria exhibit N-acetyl transferase

activity which would remove octopamine from the incubation medium by converting it to N-acetyloctopamine which is inactive (Evans, 1980). In the study of Mir and Vaughan, (1981) it was shown that if intact thoracic ganglia from S. gregaria were incubated with [^{3}H] tyrosine,radioactivity could be recovered in octopamine, although the radioactivity in N-acetyloctopamine was 3 times greater. A more direct indication of the ability of intact tissue (from S. gregaria) to metabolize octopamine was provided in the study of Goosey and Candy (1982). It was shown that an intact malpighian tubule could completely remove 13 nmoles of octopamine from the incubation medium (0.6 ml) within 1 hour. As was shown by Mir and Vaughan, (1981) intact nervous tissue possesses considerable N-acetyl-transferase activity and in a direct comparison between the activity of N-acetyltransferase of homogenized cerebral ganglia and malpighian tubules, it was observed that the activity of cerebral ganglia was almost 4 times greater, (Hayashi et al, 1977). This would indicate that intact cerebral ganglia should remove octopamine at a higher rate than malpighian tubules. In the present study intact cerebral ganglia (4) were incubated in a volume of 0.5 ml with 50 nmoles of octopamine. If the rate of removal of octopamine by intact cerebral ganglia was at least equal to that of malpighian tubules,(13 nmoles/hour) then octopamine would have been completely removed within the first 4 hours, if not before. No octopamine would have remained after 22 hours. In S. gregaria at least,it appears as though the secondary stimulation of protein phosphorylation by octopamine is due to a long-term effect stimulated by its initial presence.

No data are available for the metabolism of octopamine by tissues of

Aplysia, although Aplysia abdominal ganglion, like the cerebral ganglion of S. gregaria contains endogenous octopamine (35 ng/g wet weight of tissue) and possibly mechanisms for its removal, (Robertson & Juorio, 1976).

4:4:2 Homogenized Tissues

In homogenates of S. gregaria cerebral ganglia octopamine increases the level of cyclic AMP, (Kilpatrick et al, 1980) and cyclic AMP increases protein phosphorylation, (Figure 23). Octopamine also increases protein phosphorylation in intact cerebral ganglia and should therefore increase protein phosphorylation in homogenized ganglia. However, no effect of octopamine on protein phosphorylation was detected under the conditions in which an increase in the level of cyclic AMP was observed by Kilpatrick et al, (1980) or the increase in protein phosphorylation in response to cyclic AMP, (Tables 10 and 11). Before octopamine could have an effect on protein phosphorylation it would first have to stimulate the production of cyclic AMP. The high concentrations of ATP (500 μM) used in adenylate cyclase assays, (Kilpatrick et al, 1980) were too high as protein phosphorylation was maximal and even added cyclic AMP (10^{-5} M) had no further effect at these concentrations, (Figure 30). The low concentrations of ATP (10 μM) used in the protein phosphorylation assays were presumably too low for cyclic AMP production.

An octopamine stimulated increase in the phosphorylation of total protein was observed when homogenates were pre-incubated with unlabelled ATP and octopamine for 5 minutes prior to the addition of [γ^{32}P] ATP. This presumably occurs because during the pre-incubation with unlabelled ATP, sufficiently high levels of cyclic AMP were produced. The [γ^{32}P] ATP added after 5 minutes was then mainly used up in cyclic AMP-dependent protein phosphorylation.

If [γ^{32}P] ATP was present throughout it would also have been used up in the production of cyclic AMP, therefore, less would have been available for protein phosphorylation. Incubation of homogenate with octopamine and [γ^{32}P] ATP did actually result in a lower incorporation of ^{32}P into protein when compared to control incubations, (Figure 30).

Octopamine stimulated the phosphorylation of 3 specific proteins (M_r 51,000; 39,000 and 21,000) in homogenates of S. gregaria cerebral ganglia, (Figure 41). It is not certain why octopamine stimulated an increased phosphorylation of the M_r 51,000 and 21,000 proteins in homogenates whereas only phosphorylation of the M_r 39,000 protein was increased in intact ganglia. Presumably this occured because in intact tissue the protein kinase did not have access to the other 2 proteins.

The phosphorylation of the 3 proteins (M_r 51,000; 39,000 and 21,000) was influenced by agents which are involved in the stimulation of adenylate cyclase. The phosphorylation of these proteins by octopamine was not as effective as octopamine in the presence of GTP. Guanylyl 5'-imidodiphosphate (GppNHp) in the absence of octopamine also stimulated the phosphorylation of these proteins. This is in agreement with the effect of these compounds on adenylate cyclase activity in the central nervous systems of S. gregaria and other invertebrates. The stimulation of adenylate cyclase activity by octopamine is not as effective as in the presence of GTP and GppNHp can stimulate activity in the absence of octopamine. This has been shown

to occur in homogenates of S. gregaria cerebral ganglia, cockroach (P. americana) brain and horsehoe crab (Limulus polyphemus) brain, (Kilpatrick, 1981; Harmar & Horn, 1977; Atkinson et al, 1977). This is further evidence that the octopamine-stimulated protein phosphorylation in S. gregaria CNS is mediated by cyclic AMP.

Fluoride ion directly stimulates adenylate cyclase in the absence of hormone or guanine nucleotides. In avian erythrocyte 10 mM sodium fluoride alone is 144 and 26 times more potent in stimulating cyclase activity than either isoprenaline or GppNHp respectively, (Aurbach et al,1975). Fluoride (10 mM) also stimulates a 10.3 fold increase in cyclase activity from L. polyphemus CNS, (Atkinson et al, 1977).

Fluoride has also been shown to inhibit protein phosphatase from the CNS of the tobacco hornworm (Manduca sexta) and rat brain (Albin & Newburgh, 1975; Maeno & Greengard, 1972). The activation of adenylate cyclase and the inhibition of protein phosphatase by fluoride should allow it to act synergistically with agents that stimulate protein phosphorylation which is mediated by cyclic AMP. However, at a concentration of 10 mM it significantly decreased the octopamine-stimulated protein phosphorylation in homogenates of S. gregaria cerebral ganglia, (Table 12). It has been shown that this concentration inhibits protein kinase from mammalian brain but is without effect on cyclic AMP-dependent protein phosphorylation in synaptosomes from Drosophila, (Rodnight et al, 1975; Kelly, 1981). It seems that the predominant effect of fluoride in S. gregaria CNS under the conditions used in the present study, is the inhibtion of protein kinase.

4:5 Cyclic AMP-stimulated Protein Phosphorylation

The acceptor proteins for kinases are classified as intrinsic when an endogenous protein substrate is phosphorylated, or extrinsic when exogenous proteins such as casein, phosvitin histone or protamine are included in the assay of kinase activity. In the present study only intrinsic protein phosphorylating (kinase) activity was studied.

Intrinsic protein kinase activity has a cyclic AMP-insensitive component (basal) also, i.e. the incorporation of ^{32}P into protein occurs in the absence of cyclic AMP. In the present study basal protein phosphorylation acted as control for cyclic AMP (and other agents) dependent protein phosphorylation.

4:5:1 Comparison of Cyclic AMP-dependent Protein Kinase Activity of S. gregaria CNS with that of Mammalian CNS

Cyclic AMP-dependent protein kinase activity in S. gregaria cerebral ganglion appears to be similar to that in mammalian brain. In homogenates of S. gregaria cerebral ganglia cyclic AMP increases kinase activity to a maximum after which activity is inhibited at concentrations greater than 50 μM, (Figure 23). The concentration of cyclic AMP which gives half-maximal stimulation is 2.5 μM. This is in agreement with the effect of cyclic AMP on kinase activity of guinea-pig brain. A significant inhibition of activity occurs with a cyclic AMP concentration of 500 μM and a half-maximal stimulation is achieved with 1 μM, (Rodnight, 1975; Rodnight et al, 1975).

Increasing the incubation temperature from 22 ^{o}C to 37 ^{o}C did not have a significant effect on either cyclic AMP-dependent or basal protein kinase activity in S. gregaria, (Figure 25). In guinea-pig brain, however, kinase activity is significantly less (approximately 5 fold) at 20 ^{o}C than it is at 37 ^{o}C both in the absence or presence of cyclic AMP, (Rodnight et al, 1975). It is not certain why S. gregaria kinase activity is not affected by temperature over this range, although it may reflect the fact that S. gregaria is a poikilothermic animal. It may be that S. gregaria protein kinase has a wider temperature range over which activity is unaffected. Unlike mammalian kinases which are not subjected to variations in temperature in vivo due to a constant body temperature, the kinase from S. gregaria would have to function over a wide range. Clearly this is useful from a survival point of view. However, the activity of other enzymes is affected by temperature over this range in S. gregaria. Octopamine-stimulated adenylate cyclase activity is less at 22 ^{o}C compared to activity at 30 ^{o}C, (Kilpatrick, 1981). It is confusing then why the kinase should have a wide temperature range and not adenylate cyclase.

S. gregaria protein kinase activity had a pH optimum between 7 and 8 both in the presence and absence of cyclic AMP, (Figure 26). The change in phosphorylation on changing the pH in the presence or absence of cyclic AMP is similar to that observed for mammalian kinases which in guinea-pig cerebral membrane fragments has a pH optimum of 7.2-7.4, (Rodnight et al, 1975; Weller, 1979).

No kinase activity can be measured from membrane fragments of bovine brain in the absence of magnesium, (Miyamoto et al, 1969). High concentrations (in the order of 10 μM) result in a significant inhibition of the cyclic AMP-stimulated increase in activity, and is completely inhibited at 30 mM, (Rodnight et al, 1975). It is generally useful to have an excess of magnesium salt over ATP as the Mg ATP^{-2} (the species of Mg ATP required by the kinase) concentration varies greatly if the total magnesium salt and ATP concentrations are varied in equimolar proportions. If the magnesium salt is in excess, the proportion of ATP present as Mg ATP^{2-} will be constantly high. At an ATP concentration of 10 μM and 2 mM-$MgCl_2$, 89.2% of the ATP is present as Mg ATP^{2-}, (Storer & Cornish-Bowden, 1976). The effect of Mg^{2+} concentration on S. gregaria kinase activity was not studied although the $MgSO_4$ concentration was always 2 mM with 10 μM ATP. This would also have yielded on Mg ATP^{2-} proportion of 89.2% as Mg^{2+} as the chloride, acetate, or sulphate salt are equally effective, (Rodnight et al, 1975).

The time course of protein phosphorylation was studied primarily to ascertain when the greatest stimulation by cyclic AMP occured. The absolute time course of protein phosphorylation is a balance between 3 main factors;

1) Kinase activity; the rate at which phosphate is transferred to protein acceptors.
2) Phosphatase activity; the rate at which phosphate is removed from the protein acceptors.
3) The concentration of ATP; as the ATP is used up the rate

of phosphorylation decreases and the rate of dephosphorylation increases and consequently the net amount of phosphorylated protein decreases.

A net increase in the phosphorylation of protein is observed because kinase activity predominates over phosphatase activity. It has been shown that in mammalian brain, kinase activity is responsible for the initial level of protein-bound phosphate as the phosphatase acts at a much lower rate. This effect is not due to a low level of substrate for the phosphatase until the kinase has transferred phosphate to the protein. The phosphatase acts at a lower initial rate than the kinase even when fully phosphorylated protein is the substrate, (Weller, 1974). Cyclic AMP increases the rate of kinase activity (the initial rate of phosphorylation) and does not affect the rate of phosphatase activity, (Weller, 1974; Rodnight et al, 1975). In homogenates of S. gregaria cerebral ganglia, cyclic AMP increased the rate of protein phosphorylation and a maximum was achieved after 60 seconds, although the maximum for basal protein phosphorylation occured after only 15 seconds, (Figure 29). The incorporation of ^{32}P into protein from synaptic membrane fragments of rat cerebral cortex incubated with 5 μM [$\gamma^{32}P$] ATP appears to occur rapidly. A plateau is reached after only a few seconds both in the presence and absence of cyclic AMP, (Ueda et al, 1973). It has been claimed that this effect with such a low concentration of ATP is an artifact due to rapid hydrolysis (within the first few seconds) of the ATP which would prevent any further protein phosphorylation, (Weller & Morgan, 1976). The important observation in the study of Ueda et al,

(1973), however, was that even after 2 minutes the ^{32}P incorporated into protein was much greater in cyclic AMP preparations than in control preparations. It appears that if it is intended to study whether various agents increase or decrease protein phosphorylation as in the present study, then provided that incubation times are not excessive, low concentrations of ATP are adequate. Low concentrations of ATP (in the range 3-20 μM) have been successfully employed by many workers in the study of the effect of cyclic nucleotides on protein phosphorylation in nervous tissue, (Johnson et al, 1971; Routtenberg & Ehrlich, 1975; Talmadge et al, 1975; Bandle & Levitan, 1977; De Blas et al, 1979; Kelly, 1981). In the present study the concentration of ATP was kept constant at 10 μM (except where specified) as this allowed the maximum stimulation by cyclic AMP, i.e. the greatest difference between basal and cyclic AMP preparations, (Figure 30), (Rodnight et al, 1975).

Most tissue fractions contain cyclic AMP phosphodiesterase, both a soluble and particulate activity have been reported, (Appleman et al, 1973), therefore, phosphodiesterase inhibitors are commonly employed in protein kinase assays to prevent the hydrolysis of cyclic AMP. Theophylline and caffeine strongly inhibit intrinsic protein kinase activity of synaptic membrane fragments from ox brain, as much as 50% inhibition is observed with a theophylline concentration of 10 μM, (Weller, 1979), however, the stimulation by cyclic AMP is unaffected, (Weller & Rodnight, 1973). In the present study 10 mM theophylline was routinely employed in all incubations of homogenized tissue.

4:5:2 Cyclic AMP-dependent Protein Phosphorylation in Subcellular Fractions

The effect of cyclic AMP on protein phosphorylation was studied in subcellular fractions prepared by 2 different methods;

1) Homogenization of tissue in hypoosomotic buffer (which should have resulted in lysis of both the plasma membrane and organelle membranes) followed by centrifugation to yield a soluble (Sf) and particulate (Pf) fraction.
2) Gentle homogenization of the tissue in 0.25 M sucrose (which should have allowed the plasma membrane to rupture and the organelles to remain intact) followed by classical differential centrifugation methods to obtain subcellular fractions such as mitochondrial, synaptosomal and microsomal, (Birnie, 1972).

Cyclic AMP increases the incorporation of ^{32}P from [$\gamma^{32}P$] ATP into protein from various subcellular fractions of rat cerebral cortex. Cyclic AMP increases protein phosphorylation in the nuclear, mitochondrial, microsomal and soluble fractions, in the presence of both exogenous and endogenous protein kinase, (Johnson et al, 1971). The greatest effect of cyclic AMP being in synaptic plasma membranes and the microsomal fraction, (Johnson et al, 1971). Cyclic AMP-dependent increases in total protein phosphorylation in subcellular fractions from S. gregaria cerebral ganglia were also detected under similar incubation conditions in a crude nuclear fraction, (Figure 39) crude mitochondrial fraction, (Figure 39) and soluble fraction of tissue homogenized in either 0.25 M sucrose, (Figure 52) or 6 mM Tris-maleate, (Figure 33). A very small increase in the

presence of cyclic AMP was detected in the Pf, (Figure 33). Almost all of the cyclic AMP-dependent protein kinase activity of S. gregaria unlike that of mammalian protein kinase, appears to reside in the Sf. The Sf and Pf were, however, prepared by homogenizing the tissue under hypoosomotic conditions and centrifuging to separate membrane fragments from soluble components. It is possible that these hypoosomotic conditions result in solubilization of most of the cyclic AMP-dependent protein kinase from the membrane and is, consequently, removed with the Sf. When the crude homogenate is incubated with [γ^{32}P] ATP before separating into Sf and Pf a cyclic AMP-dependent increase in phosphorylated protein can be detected in the Pf, (Figure 33) suggesting that protein substrate is present in the Pf.

It appears that comparatively less cyclic AMP-dependent protein phosphorylation occurs in invertebrate nervous tissue. In nervous tissue from garden snail, (Helix pomatia) cyclic AMP stimulates an increased protein phosphorylation in 20,000 g and 150,000 g supernatants, but not in the respective pellet (particulate) fractions, (Bandle & Levitan, 1977). Cyclic AMP markedly stimulates histone kinase activity in the 20,000 g supernatant but has very little effect on the 20,000 g pellet. If phosphorylation is carried out in the 700 g supernatant prior to centrifugation cyclic AMP stimulates the phosphorylation of protein which separates into both 20,000 g supernatant and pellet indicating a particulate substrate is present, although the cyclic AMP-dependent kinase may be soluble. Similar results

have obtained in Aplysia nervous tissue. Cyclic AMP stimulates the endogenous phosphorylation of 10 proteins (M_r 21,000-130,000) in 12,000 g supernatants, but no cyclic AMP-dependent phosphorylation is detected in the 12,000 g pellets, (Novak-Hofer & Levitan, 1981). However, the cyclic AMP-dependent phosphorylation of an M_r 118,000-120,000 protein can be detected in 15,000 g pellets prepared from intact Aplysia nervous tissue incubated with ^{32}Pi, (Levitan et al, 1974). This suggests that in Helix and Aplysia the cyclic AMP-dependent protein kinase is either soluble or easily solubilized. In S. gregaria the lack of effect of cyclic AMP on total protein phosphorylation in the Pf also appears to be due to solubilization of the kinase, although some kinase activity remains as cyclic AMP stimulates the phosphorylation of specific proteins in the Pf, (Figures 47 and 50). It appears that if a particulate-bound kinase is present in S. gregaria CNS then it is more readily removed in hypoosmotic buffer (6 mM Tris/maleate) than in 0.25 M sucrose. Cyclic AMP-dependent protein kinase activity is also present in 30,000 g pellets from Drosophila nervous tissue homogenized in 0.25 M sucrose, (Kelly, 1981).

In mammalian nervous tissue most of the cyclic AMP-dependent protein kinase activity occurs in synaptic plasma membranes which are prepared by lysing synaptosomes in hypoosmotic buffer prior to density gradient centrifugation, (Johnson et al, 1971; Rodnight et al, 1975; Greengard, 1978; Weller; 1979). This indicates that mammalian cyclic AMP-dependent protein kinase is strongly bound to membranes and not easily solubilized. It appears then that the solubility of cyclic AMP-dependent protein kinase in different

species is in the following order; molluscs > insects > mammals.

Invertebrate nervous tissue does not appear to contain the 2 synaptosomal cyclic AMP-stimulated phosphoproteins Synapsin 1a and 1b (M_r 86,000 and 80,000) present in mammalian CNS, (Greengard, 1978; Nestler & Greengard, 1983). In both Aplysia and Helix nervous tissue, cyclic AMP stimulates the phosphorylation of a single protein (M_r 118,000-120,000) in 15,000 g and 20,000 g pellets respectively, (Levitan et al, 1974: Bandle & Levitan, 1977). The phosphorylation of 3 proteins (M_r 160,000; 74,000 and 31,000) is increased and the phosphorylation of 1 protein (M_r 47,000) is decreased in response to cyclic AMP in 30,000 g pellets from Drosophila nervous tissue, (Kelly, 1981). The phosphorylation of many proteins occurs in 25,000 g pellets in S. gregaria nervous tissue in response to cyclic AMP,none of which have an M_r 80,000-86,000, (Figure 52).

The protein which exhibits the greatest cyclic AMP-stimulated phosphorylation in S. gregaria cerebral ganglia is the M_r 39,000 protein, (Figure 45). The cyclic AMP-stimulated phosphorylation of this protein occurs in the Sf, Pf, 25,000 g fractions and 150,000 g supernatant (Figures 49, 50, 51 and 52). It is not certain if the M_r 39,000 band consists of one protein only or two different proteins of the same molecular weight, one of which is soluble and the other membrane-bound. A similar effect is observed in Helix nervous tissue, where cyclic AMP stimulates the phosphorylation of an M_r 118,000-120,000 protein in both 20,000 g pellets and supernatants, (Bandle & Levitan, 1977).

4:6 Physiological Actions in which Octopamine and Cyclic AMP-stimulated Protein Phosphorylation is Possibly Involved

In the present study protein phosphorylation was not correlated to any of the physiological effects which octopamine or cyclic AMP have on insect nervous tissue. This section therefore discusses the changes in protein phosphorylation stimulated by octopamine and cyclic AMP in the context of the physiological actions in which they are involved.

4:6:1 Octopamine-stimulated Na^+ Uptake

Na^+ transport in insect nerve-cord appears to be regulated by octopamine. The uptake of Na^+ into intact nerve-cord from P. americana is increased by octopamine, (Steele & Chan, 1980). It is thought that this occurs because octopamine increases the permeability of the apical membrane of the perineurial cells to Na^+. This has been confirmed by direct measurement of Li^+ uptake in the absence and presence of octopamine, (Steele & Chan, 1980).

Na^+ transport in neuronal and non-neuronal tissues is associated with changes in protein phosphorylation and the injection of catalytic subunit of cyclic AMP-dependent protein kinase into neuronal tissue increases the entry of other ions such as K^+ and Ca^{2+}, (Section 4:6:3). In toad-bladder antidiuretic hormone (ADH) increases both the membrane permeablility to Na^+ and the level of cyclic AMP. This is accompanied by a decrease in the phosphorylation of 2 membrane proteins (M_r 54,000 and 37,000). Monobutyryl cyclic AMP mimics the effects of ADH, (Walton et al, 1975). This suggests that Na^+ uptake into toad-bladder occurs by a process involving a cyclic AMP-dependent decrease in protein phosphorylation. A decrease

in protein phosphorylation is also thought to be involved in Na^+ uptake in nervous tissue from the shore crab (Carcinus maenas). High molecular weight proteins (M_r 380,000-530,000) termed sodium conductin, which are associated with the sodium permeation sites and are thought to be components of the Na^+-channel, undergo a cycle of phosphorylation-dephosphorylation that is influenced by neurotropic compounds and inorganic ions, (Schoffeniels, 1980). The net phosphorylation of sodium conductin is decreased by Na^+ ions, increased by K^+ ions and compounds which increase Na^+ entry such as veratradine also decrease the net phosphorylation and compounds which decrease Na^+ entry such as tetrodotoxin increase it, (Schoffeniels & Dandrifosse, 1980).

It is not known if octopamine stimulates Na^+ uptake in S. gregaria CNS, but presumably it does as its structure is similar to that of P. americana and octopamine has similar effects on both tissues, (Bullock & Horridge, 1965; Evans, 1980). It would appear then that the octopamine-stimulated phosphorylation of the M_r 39,000 protein in S. gregaria CNS is not involved in Na^+ entry as an increase and not a decrease in phosphorylation was observed. However, the possibility that it is involved cannot be ruled out merely on the basis of the trend of decreased phosphorylation associated with Na^+ uptake in toad-bladder and C. maenas nervous tissue. In turkey erythrocytes Na^+ entry is associated with a cyclic AMP-mediated increase in the phosphorylation of an M_r 240,000 protein, (Rudolph et al, 1978). More data correlating Na^+ uptake with protein phosphorylation in S. gregaria is obviously neccessary before any firm conclusions can be drawn, although this at least indicates that there may be a link.

4:6:2 Protein Phosphorylation and Octopamine-stimulated Glycogenolysis

Octopamine is released in response to stress in insects. Both mechanical stress (tumbling in a rotating drum) and increasing the ambient temperature from 25 ^{o}C to 45 ^{o}C result in a two-fold increase in the level of octopamine in haemolymph from S. gregaria and cockroach, (Periplaneta americana), (Davenport & Evans, 1984). It appears that the released octopamine is involved in the mobilization of energy stores, particularly in the nervous system. Nerve-cord glycogen in P. americana decreases by 33% within 2 hours of the intra-abdominal injection of 25 n moles of octopamine, and incubation of the isolated nerve-cords with octopamine results in a 48% increase in phosphorylase activity. This latter effect is potentiated be caffeine and mimicked by cyclic AMP, (Robertson & Steele, 1972; Robertson & Steele, 1973). Coupled with the observation that octopamine stimulates an increase in the level of cyclic AMP in P. americana nerve-cord, (Nathanson & Greengard, 1973), suggests that the glycogenolysis in response to octopamine occurs via a cyclic AMP-dependent activation of glycogen phosphorylase.

It is well established (at least in rabbit skeletal muscle) that the enzymes involved in glycogenolysis are activated by cyclic AMP-dependent phosphorylation, (Cohen, 1973; Cohen & Antoniw, 1973; Busby & Radda, 1976). Glycogen phosphorylase from all tissues so far examined including locust fat body, (Applebaum & Schlesinger, 1973) consists of 2 forms; a relatively inactive b form and an active a form, (Busby & Radda, 1976). Activation of phosphorylase b by conversion

to the a form is achieved by phosphorylation of the b form. This is catalysed by phosphorylase kinase which is itself activated by being phosphorylated by a cyclic AMP-dependent protein kinase, (Cohen, 1973).

Considerable reserves of glycogen are present in S. gregaria nervous tissue, being almost 6-fold greater than in rat cerebral cortex, (Clement & Strang, 1978). Although it has not been unequivocally demonstrated that octopamine stimulates phosphorylase activity in nervous tissue from S. gregaria. It presumably can do so, as the other effects of octopamine are similar in both S. gregaria and P. americana, (Evans, 1980). It may be possible then that the octopamine-stimulated phosphorylation of the M_r 39,000 protein in intact cerebral ganglia from S. gregaria is involved in some aspect of glycogenolysis. If it is involved in glycogenolysis it does not appear to be at the level of glycogen phosphorylase as glycogen phosphorylase exists as a dimer of molecular weight 185,000 which can be dissociated into monomers of 95,000-100,000 in almost all tissues, (Weller, 1979). Monomers isolated from the flight muscle of the blowfly, (Phormia regina) have a molecular weight of 97,000, (Childress & Sacktor, 1970). This is close to the value of 92,500 obtained for rabbit skeletal muscle, (Seery et al, 1967) suggesting that the structure of glycogen phosphorylase is well conserved.

Phosphorylase kinase has been demonstrated in insects. It is present in flight muscle from P. regina and silkmoth, (Hyalophora cecropia) and requires Pi for activity, which indicates that

it is activated by phosphorylation, (Sacktor et al, 1974; Ashida & Wyatt, 1979). However, information about the subunit structure of insect phosphorylase kinase is not presently available. Phosphorylase kinase from rabbit skeletal muscle consists of 4 different subunits; α, β, γ and δ with the holoenzyme structure being $(\alpha\beta\gamma\delta)_4$, (Weller, 1979). The δ subunit has been identified as calmodulin, (Cohen et al, 1978) and the molecular weights of the α, β and γ subunits have been estimated to be 118,000, 108,000 and 41,000 respectively, (Hayakawa et al, 1973). The only subunit of phosphorylase kinase from rabbit skeletal muscle with a similar molecular weight to the M_r 39,000 octopamine-stimulated phosphoprotein from S. gregaria CNS is the γ subunit. However, in the activation of rabbit phosphorylase kinase, the γ subunit does not become phosphorylated, only the α and β subunits are phosphorylated, (Cohen, 1973). Without information on the structure of insect phosphorylase kinase it is not possible to draw a firm conclusion about whether or not the octopamine-stimulated M_r 39,000 phosphoprotein is involved in glycogenolysis. It could,however, possibly be involved in some aspect of glycogenolysis which is without direct analogy in mammalian tissues.

4:6:3 Possible Involvement of Protein Phosphorylation in the Generation of Electrical Potentials

The electrophysiological events involved in neurotransmission are rapid, typically in the millisecond range, (Phillis, 1970). It appears, therefore, that rapid phosphorylation would have to be a prerequisite for the involvement of protein phosphorylation in the generation of electrical potentials.

The incubation of intact tissues with putative neurotransmitters result in a comparatively slow increase in protein phosphorylation. The maximum effect of noradrenaline on the phosphorylation of protein from respiring slices of guinea-pig cerebral cortex is seen after 6 minutes, (Williams & Rodnight, 1976). The application of electrical pulses, however, results in a maximal increase in phosphorylation within 2 seconds, (Williams & Rodnight, 1975). The difference in time course is thought to be related to the time required for exogenously applied noradrenaline to diffuse to its site of action. Electrical pulses presumably stimulate the release of endogenous noradrenaline at, or near its site of action, as adrenergic β-receptor blocking agents such as practolol and ICI 66082 prevent the increased protein phosphorylation in response to both noradrenaline and electrical pulses, (Williams & Rodnight, 1976).

The time course of the increase in protein phosphorylation in response to electrical pulses is similar to that of the electrophysiological response to iontophoretically applied

noradrenaline. The iontophoretic application of noradrenaline to cerebral neurones exhibits a latency of onset of electrophysiological response ranging from a few milliseconds to several seconds, (Phillis, 1971; Salmoiraghi, 1966) and may then persist for up to 5 minutes, (Salmoiraghi, 1966).

It is possible that the octopamine stimulated protein phosphorylation in S. gregaria cerebral ganglion is also involved in neurotransmission. Stimulation of the M_r 39,000 protein is observed after 10 minutes exposure to octopamine which as described previously for mammalian nervous tissue, is probably the time required for it to diffuse to its site of action, although shorter times were not studied. This response after 10 minutes is within the time course of electrophysiological events in invertebrate nervous systems. The inhibitory and excitatory junction potentials from molluscan neurones can be either short duration lasting up to 400 milliseconds or long duration lasting from 20-60 seconds or longer, (Phillis, 1970). It has also been observed that a rise in the level of cyclic AMP (which is thought to mediate protein phosphorylation in response to octopamine) in intact nervous tissue from Aplysia is only detected after at least 4 minutes nerve stimulation, (Cedar et al, 1972). Cyclic AMP can stimulate protein phosphorylation in cell-free systems within a few seconds (the fastest times it is at present possible to study protein phosphorylation). The phosphorylation of Synapsin 1 in response to cyclic AMP reaches a maximum within 5 seconds, (Greengard, 1976), and an appreciable cyclic AMP-stimulated protein phosphorylation (including the M_r 39,000 protein)

occurs in homogenates of S. gregaria cerebral ganglia within 10 seconds, (Figure, 44).

Many recent studies have provided direct evidence that cyclic AMP-dependent protein phosphorylation is involved in the generation of electrical potentials. Injection of the catalytic subunit of cyclic AMP-dependent protein kinase and protein kinase inhihbitor into invertebrate neurones result in changes in ion channel permeability. The results of various studies are summarized in Table 14.

Table: 14 Ion channels regulated by protein phosphorylation in various cells

Cell studied	Parameter tested	Reagent	Change induced by protein phosphorylation
Aplysia bag	Action potential duration	C subunit	Ca^{2+}-dependent K^+ channel inhibited
Aplysia sensory	Action potential duration	C subunit and PKI	Novel K^+ channel inhibited
Myocytes	Voltage-clamp currents	C and R subunits	Ca^{2+} channel activated
Hermissenda photoreceptors	Voltage-clamp currents	C subunit	Delayed K^+ channel inhibited
Helix neurons	Voltage-clamp currents	C subunit	Ca^{2+}-dependent K^+ channel activated
Aplysia R15	Voltage-clamp currents	PKI	Inwardly rectifying K^+ channel activated

Reagents; C- catalytic subunit of cyclic AMP-dependent protein kinase
PKI- protein kinase inhibitor

from Levitan et al, 1983

4:6:4 A Possible Role for Protein Phosphorylation in Memory

It has been proposed that changes in the phosphorylation of certain proteins could be involved in memory and learning processes. Changes in protein phosphorylation following learning experiences have been demonstrated in both mammalian and invertebrate CNS. These changes in protein phosphorylation accompany changes in ionic permeability which are associated with behavioural sensitization and habituation.

An increase in the incorporation of ^{32}P from [$\gamma^{32}P$] ATP into proteins from the membrane fraction of brain can be detected in rats and mice following a training experience involving foot shock at regular intervals, 24 hours prior to sacrifice. An increased incorporation of ^{32}P into 2 proteins (M_r 47,000 and 10,000-18,000) is observed, (Ehrlich et al, 1978).

The marine mollusc, (Hermissenda crassicornis) moves toward a light source as the micro-organisms on which it feeds exist in well-lit waters. If the animals are conditioned by applying light stimulus followed by rotation (simulating sea turbulance) they move toward the light only very slowly in comparison to animals which receive light stimulus and rotation in a random manner. This indicates that they had learned to associate light with rotation, (Alkon, 1983). This associative learning in Hermissenda is accompanied by changes in both ionic permeability (increased Ca^{2+} current and decreased K^+ current) and protein phosphorylation. There is an increased incorporation of ^{32}P into 2 proteins (M_r 20,000 and occasionally 25,000)when the

CNS (including the eyes) of conditioned animals are incubated with ^{32}Pi, (Neary et al, 1981).

Changes in ionic permeability have been identified as being involved in memory. Depression in the gating of K^+ channels results in an increased Ca^{2+} influx which enhances transmitter release. This mimics the physiological changes that accompany behavioural sensitization, a simple form of short-term memory, (see Section 1:1). The injection of catalytic subunit of cyclic AMP-dependent protein kinase into Aplysia nerves (which is thought to, either phosphorylate components of the K^+ channel itself, or a modulatory protein which acts on the channel) also depresses the K^+ current which would normally oppose the Ca^{2+} current. This results in an increased Ca^{2+} influx and, consequently, increased neurotransmitter release, (Castellucci et al, 1980). Similar results have been obtained in Hermissenda where increased activity of calcium-dependent protein kinase (which is also thought to be involved in the control of K^+ permeability) in response to repeated stimuli causes increased excitability (sensitization), (Alkon, 1983).

It was interesting to note that octopamine stimulated the phosphorylation of 2 proteins (M_r 21,000 and 25,000) after 22 hours but not after 10 minutes, (Figures20, 21 and 22). These 2 phosphoproteins have a similar molecular weight to the 2 phosphoproteins (M_r 20,000 and 25,000) which are present in Hermissenda CNS after associative learning, (Neary et al, 1981). It is possible (merely from a speculative point of view) that

these 2 proteins are involved in memory in S. gregaria.

Sensitization and habituation occur in response to repeated stimuli which would result in a constant release of neurotransmitter. Incubation of intact ganglia with octopamine (a putative neurotransmitter) for a long period (in the order of a few hours) may be analogous to the release of neurotransmitter in response to repeated stimuli.

4:7 Calcium-dependent Protein Phosphorylation

Calcium increased total protein phosphorylation in homogenates of S. gregaria cerebral ganglia and a maximum was achieved with 1 mM $CaCl_2$ in the presence of 0.7 mM EGTA, (Figure 31). In the present study EGTA was present in all experiments in order to reduce the effect of endogenous Ca^{2+} as basal phosphorylation in the absence of EGTA was much higher, (Figure 34). $CaCl_2$ (0.5 mM) in the presence of EGTA (0.2 mM) stimulates the phosphorylation of many proteins from rat synaptosomes (Schulman & Greengard, 1978a, b). Although calcium stimulates the phosphorylation of many proteins in rat synaptosomes, when the synaptosomal membranes are separated from their cytosol prior to incubation, calcium no longer has any effect, (Schulman & Greengard, 1978a). Calcium-dependent protein phosphorylation can be regained by the addition of boiled cytosol or calmodulin to the membranes, (Schulman & Greengard, 1978b). In S. gregaria nervous tissue the calcium-dependent phosphorylation of membrane-bound proteins also required the addition of exogenous calmodulin as no calcium stimulated protein phosphorylation was detected in the Pf when calcium alone was added. Calcium alone could, however, stimulate the phosphorylation of the 3 proteins (M_r 62,000; 54,000 and 31,000) in crude homogenates indicating that endogenous calmodulin was present, (Figure 50).

Calcium/calmodulin-dependent protein phosphorylation has also been demonstrated in nervous tissue from Drosophila and Aplysia. Calcium/calmodulin stimulates the phosphorylation of 4 proteins

(M_r 55,000; 51,000; 42,000 and 31,000) in crude synaptosomes from Drosophila, (Kelly, 1981). Calcium on its own stimulates the phosphorylation of a protein (M_r 21,000) in 12,000 g pellets from Aplysia nervous tissue which is inhibited by trans- flupenthixol, indicating that calmodulin-stimulated calcium-dependent protein kinase is involved, (Novak-Hofer & Levitan, 1981).

Two of the calcium/calmodulin-stimulated phosphoproteins from rat synaptosomes are present in the membranes of synaptic vesicles. It is thought that these 2 proteins (M_r 51,000-54,000 and 62,000-63,000) termed DPH-M and DPH-L are involved in the release of neurotransmitter as the depolarization-dependent uptake of calcium, neurotransmitter release and phosphorylation of these proteins occur simultaneously, (De Lorenzo et al, 1979). It is possible that the calcium-dependent phosphoproteins (M_r 62,000 and 54,000) from S. gregaria cerebral ganglia may be similar to the proteins DPH-M and DPH-L from rat synaptic vesicles on the basis that they are particulate, have the same molecular weight and are stimulated by calcium in both tissues.

4:8 Cyclic GMP-dependent Protein Phosphorylation

Cyclic GMP stimulated the increased phosphorylation of only one protein (M_r 45,000) in homogenates of S. gregaria cerebral ganglia, (Figure 48). Cyclic AMP also stimulated the phosphorylation of an M_r 45,000 protein (possibly the same protein), although the basal phosphorylation of this protein was also high, (Figure 45D). A few proteins, the phosphorylation of which is increased by cyclic GMP, have been reported to be present in mammalian tissues and a feature of these proteins is that cyclic AMP also affects their phosphorylation, (Greengard, 1978). Cyclic GMP stimulates the phosphorylation of only one protein (M_r 23,000) from rabbit cerebellum and one protein (M_r 49,000) from mouse brain, (Schlichter et al, 1978; Malkinson, 1975). Cyclic AMP also increases the phosphorylation of the M_r 23,000 protein from rabbit cerebellum but decreases the phosphorylation of the M_r 49,000 protein from mouse brain. Both of these proteins are cytosolic and no cyclic GMP-stimulated protein phosphorylation is detected in the respective membrane fractions, (Schlichter et al, 1978; Malkinson, 1975). However, in Aplysia nervous tissue, cyclic GMP stimulates the phosphorylation of 3 proteins (M_r 92,000; 68,000 and 62,000) in membrane fractions. Cyclic AMP also stimulates the increased phosphorylation of these 3 proteins although much higher concentrations are required and unlike mammalian nervous tissue the maximal stimulation by cyclic AMP is not as great as the cyclic GMP-stimulated maximum, (Ram & Ehrlich, 1978).

The effect of cyclic GMP in the present study was investigated in the presence of EGTA. The absence of calcium may have prevented the cyclic GMP-dependent phosphorylation of other proteins as

the cyclic GMP-dependent stimulation of the M_r 49,000 protein from mouse brain required calcium, (Malkinson, 1975).

4:9 The Effect of Protein Kinase Inhibitor on Protein Phosphorylation

Two types of protein kinase inhibitor (PKI) have been described and are commonly designated type I and type II. The type I PKI from rabbit skeletal muscle (that described by Walsh et al, 1971) has an M_r 10,000-12,000 when estimated by polyacrylamide/SDS gel electrophoresis, (Whitehouse & Walsh, 1982). A type II PKI (M_r 6,000-8,000) has also been isolated from rabbit skeletal muscle, (Whitehouse & Walsh, 1982).

The PKI isolated from rabbit skeletal muscle by the method of Walsh et al (1971) binds with high affinity to the catalytic subunit of cyclic AMP-dependent protein kinase and can almost completely inhibit the cyclic AMP-dependent phosphorylation of proteins from rabbit brain, (Walsh & Ashby, 1973; Ashby & Walsh, 1973). It also inhibited the the cyclic AMP-dependent phosphorylation of all proteins from S. gregaria CNS, (Figures 48, 49 and 50). In addition it also inhibited Ca^{2+}, Ca^{2+}/calmodulin and cyclic GMP-dependent protein phosphorylation. The latter results do not agree with the effect of type I PKI on protein phosphorylation in mammalian CNS. Type I and type II PKI (similar to those from rabbit skeletal muscle) have been isolated from rat cerebellum, (Szmigielski et al, 1977). The type I PKI from rat cerebellum has no effect on Ca^{2+}/calmodulin-dependent protein phosphorylation in rat synaptosomes, (Burke & DeLorenzo, 1982). The type II PKI from rat cerebellum is,however, unspecific and can inhibit cyclic nucleotide-independent protein phosphorylation also

(Szmigielski et al, 1977).

In the present study the PKI used was prepared by the method of Walsh et al (1971) and the disagreement between the effects of this PKI (assumed to be type I) on protein phopshorylation in S. gregaria and purified type I on mammalian nervous tissue may be due to the presence of both type I and type II PKI. However, it is also possible that cyclic AMP and Ca^{2+}/calmodulin-dependent protein kinases differ from those of mammalian nervous tissue and have similar binding sites for the type I PKI.

4:10 Conclusion

Octopamine stimulates protein phosphorylation in S. gregaria CNS which is mediated by cyclic AMP and cyclic AMP-dependent protein kinase. It is not certain if the octopamine and cyclic AMP-stimulated M_r 39,000 phosphorylated band is only one or more protein(s) and further characterization is necessary. Cyclic AMP-dependent protein phosphorylation in S. gregaria is similar to that in mammalian CNS except cyclic AMP stimulates the phosphorylation of a greater number of specific proteins in S. gregaria CNS.

Ca^{2+}/calmodulin-dependent protein kinase is also present in S. gregaria CNS which is membrane-bound, although it is not known which first messengers stimulate the Ca^{2+}/calmodulin-dependent protein phosphorylation.

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